Targeting EWS/FLI1 Activity in Ewing Sarcoma

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2012

To my father

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1 Summary

Ewing sarcoma (ES) is the second most frequent bone cancer in childhood. Clinically, ES appears as very aggressive osteolytic tumor with early tendency for development of metastasis. It belongs to the group of small-round-blue-cell tumors and is comprised of largely undifferentiated cells. The unique feature of this tumor is presence of the balanced t(11;22)(q24;q12) translocation in more than 85% of cases. This gene rearrangement results in the expression of a chimeric fusion protein where RNA binding domain of EWS is exchanged by the DNA binding domain of the ets transcription factor FLI1, thus generating a dysregulated transcription factor EWS/FLI1. More than 18 less represented alternative translocations involving EWS and other ets protein family members have been described since.

Extensive evidence supports the fact that EWS/FLI1 is an essential oncogenic component of ES development. Its oncogenic activity is thought to be mediated through inappropriate regulation of target genes that are crucial for the fully malignant phenotype. Apart from having transforming and tumorigenic potential, even more important is that EWS/FLI1 appears to be necessary for tumor cell maintenance. For these reasons EWS/FLI1 represents an attractive target. However, it is widely accepted that transcription factors are ‘’undruggable’’, and EWS/FLI1 as intrinsically disordered protein is not prone to direct inhibition by a small molecule in the classical sense.

No targeted agents have been routinely introduced into therapy of ES. Even though in the last few decades there has been considerable progress in both diagnosis as well as treatment of the localised disease, only 15% of patients with metastatic disease survive. Current treatment regimens of ES are not fully exploiting the fact that specific inhibition of signalling pathways is now possible. More specifically, it is not known whether specific pathway could affect the activity of EWS/FLI1.

In this thesis, we characterized PHLDA1 as a novel direct target gene whose expression is repressed by EWS/FLI1. Moreover, the role of PHLDA1 in ES was addressed in this study. Using this gene and additional specific well-characterized target genes such as NROB1, NKX2.2, and CAV1, all activated by EWS/FLI1, as a read-out system, we established a screening system that is capable of detecting compounds targeting activity and/or expression of EWS/FLI1 either

directly or indirectly. This system is an important simplification of the previously applied gene expression based high-throughput screening, represents a more targeted approach than screenings based on survival or proliferation only, and is more robust than a screening approach based on a single-gene reporter assay. We screened a small-molecule compound library enriched for FDA-approved drugs for substances that modulated the expression of EWS/FLI1 target genes. Among a hit-list of 9 well known drugs such as camptothecin, fenretinide, etoposide and doxorubicin, we also identified the kinase inhibitor midostaurin (PKC412). Subsequent experiments demonstrated that midostaurin is able to induce apoptosis in a panel of 6 ES cell lines *in vitro* and can significantly suppress xenograft tumor growth *in vivo*. These results suggest that midostaurin might be a novel drug that is active against ES cells which might act by modulating the expression of EWS/FLI1 target genes.

In the second part of this thesis, in order to identify molecular pathway(s) that may contribute to the transcriptional activity and oncogenic properties of EWS/FLI1, we used our well established screening approach and performed screening of a small library of 153 targeted inhibitors covering all major signaling pathways. We discovered PI3K inhibitors as potent modulators of EWS/FLI1 expression. This finding was confirmed in several ES cell lines and off target effects of the PI3K inhibitors was excluded by performing genetic loss of function experiments. Analysis of the EWS/FLI1 promoter region using various deletion constructs in reporter gene assays determined two 12bp minimal elements where transcription factor(s) under PI3K control is binding. Elucidating the direct link between PI3K and EWS/FLI1 is of importance, and identity of the responsible transcription factor(s) might provide novel therapeutic opportunities.

Results presented in this thesis clearly demonstrate that screening approach here established can be used for both screening for compounds effective against ES as well as for screening targeted inhibitors leading to better understanding of the biology of ES. Together it should lead to development of novel therapeutic approaches for the treatment of ES.

2 Zusammenfassung

Ewing Sarkoma (ES) ist die zweithäufigste Ursache für Knochenkrebs bei Kindern. Klinisch gesehen ist es ein sehr aggressiver osteolytischer Tumor mit einer Tendenz zur frühen Metastasierung. ES gehört zur Gruppe der kleinen, runden blauzelligen Tumore bestehend aus undifferenzierten Zellen. Die Besonderheit dieses Tumors liegt in der balancierten Translokation t(11;22)(q24;q12), die in mehr als 85% aller Fälle auftritt. Diese Genumlagerung führt zur Expression eines chimären Fusionsproteins wobei die RNA-Bindungsdomäne des EWS mit der DNA-Bindungstelle des ets-Transkriptionsfaktors FLI1 ausgetauscht ist. Dadurch entsteht der deregulierte Transkriptionsfaktor EWS/FLI1. Es gibt mehr als 18 alternative, jedoch unterrepräsentierte, Translokationen, die ebenfalls EWS oder andere ets-Familienmitglieder enthalten.

Es gibt eindeutige Hinweise dafür, dass EWS/FLI1 ein essentieller onkogener Bestandteil für die Entwicklung von ES ist. Die onkogene Aktivität besteht vor allem aus der Deregulierung von Zielgenen, die wichtig sind für die vollständige Ausprägung eines malignen Phänotyps. Neben diesen transformierenden und tumorigenen Eigenschaften scheint EWS/FLI1 eine noch wichtigere Rolle im Überleben der Tumorzellen zu spielen. Daher repräsentiert das EWS/FLI1 Protein ein attraktives therapeutisches Zielmolekül. Allerdings ist weithin akzeptiert, dass Transkriptionsfaktoren per se nicht medikamentös behandelbar sind. EWS/FLI1, ein intrinsisch ungeordnetes Protein, kann daher nicht im klassischen Sinne direkt inhibiert werden, wie etwa mit sogenannten „kleinen Molekülen“.

Es werden keine zielgerichteten Mittel routinemässig in der ES Therapie verwendet. Erst in den letzten Jahrzehnten gab es erste Fortschritte in der Diagnose und Behandlung von lokalisierten Tumoren, nur 15% der Patienten mit Metastasen überleben. Die aktuellen Behandlungsmöglichkeiten von ES schliessen noch nicht das Wissen ein, dass man spezifische Signalwege inhibieren kann. Genauer gesagt ist es noch nicht bekannt, ob bestimmte Signalwege die Aktivität von EWS/FLI1 beeinflussen.

In dieser Doktorarbeit charakterisierten wir PHLDA1 als ein neues direktes Zielgen, dessen Expression von EWS/FLI1 unterdrückt wird und charakterisieren dessen Rolle in ES. Dieses Gen und weitere spezifische, gut charakterisierte Zielgene wie NROB1, NKX2.2, and CAV1, die alle durch EWS/FLI1 aktiviert werden, nutzten wir als Komponenten für ein Screening-System, mit dem wir molekulare Verbindungen detektieren können, die die Aktivität oder Expression von

EWS/FLI1 direkt oder indirekt verändern. Dieses System ist eine wichtige Vereinfachung von früheren auf Genexpression basierenden Hochdurchsatz-Screenings. Es repräsentiert einen zielgerichteteren Ansatz als ein Screening, das nur auf der Überlebens- und Proliferationsrate beruht, und ist mehr robust als ein Screening das auf Einzelgen-Reporter-Untersuchungen beruht. Wir haben eine Bibliothek von so genannten „kleinen Molekülen“ mit vor allem FDA anerkannten Medikamenten überprüft auf Substanzen, welche die Expression von EWS/FLI1 Zielgenen verändern. Unter der Topliste von 9 gut bekannten Medikamenten, wie Camptothecin, Fenretinide, Etoposide und Doxorubicin, hatten wir auch den Kinaseinhibitor Midostaurin (PKC412) identifiziert. Weiterfolgende Experimente zeigten, dass Midostaurin *in vitro* Apoptose in 6 ES Zelllinien induzieren und in vivo das Tumorwachstum in Xenografts signifikant unterdrücken kann. Diese Resultate zeigen, dass Midostaurin ein neues Medikament werden könnte, das aktiv ist gegen Ewing Zellen und wahrscheinlich die Expression von EWS/FLI1 Zielgenen verändert.

Im zweiten Teil dieser Arbeit nutzten wir unseren etablierten Screening Ansatz um neue molekulare Signalwege zu identifizieren, die zur transkriptionalen Aktivität und den onkogenen Eigenschaften von EWS/FLI1 beisteuern, und führten ein Screening mit einer kleinen Bibliothek von 153 zielgerichtet Inhibitoren durch, die alle wichtigen Signalwege abdeckt.

Wir entdeckten PI3K Inhibitoren als potentielle Regulatoren der EWS/FLI1 Expression. Diese Erkenntnis wurde in mehreren ES Zelllinien bestätigt und „off target“ Effekte des PI3K Inhibitors wurden durch genetische „loss-of-function“ Experimente ausgeschlossen. Die Analyse der EWS/FLI1 Promoterregion durch verschiedene Deletionskonstrukte in Reportergenversuchen ermittelte zwei 12bp minimale Elemente an der Transkriptionsfaktoren unter PI3K Kontrolle binden können.

Die Aufklärung dieser direkten Verbindung von PI3K und EWS/FLI1 ist sehr wichtig, da diese neue mögliche therapeutische Möglichkeiten darstellen.

Die hier präsentierten Resultate zeigen klar, dass das etablierte Screening System für eine Untersuchung von Verbindungen oder zielgerichteten Inhibitoren, die effektiv gegen ES sind, genutzt werden kann, was zu einem besseren Verständnis der ES Grundlagen führen kann. Zusammen sollte es zu neuen Möglichkeiten von therapeutischen Ansätzen in der Behandlung von ES führen.

3 Abbreviations

AAPC	average annual percentage change
ACCIS	automated childhood cancer information system
ARA-C	cytosine arabinoside
CAV1	caveolin 1
CCND1	cyclin D1
ChIP	chromatin immunoprecipitation
CREB1	cAMP responsive element binding protein 1
CT	computed tomography
DMSO	dimethyl sulfoxide
DNA-BD	DNA binding domain
EGFR	epidermal growth factor receptor
ERK	extracellular signaling-regulated kinase
ES	Ewing sarcoma
ES/PNET	Ewing sarcoma/primitive neuroectodermal tumor
ETS	E twenty six
EWSR1	Ewing sarcoma breakpoint region 1
FLI1	Friend leukemia virus integration 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IGF1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IGFBP3	insulin-like growth factor binding protein 3
IHC	immunohistochemistry
MAPK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSC	mesenchymal stem cell

mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa b
NKX2.2	NK2 homeobox 2
NROB1	nuclear receptor subfamily 0, group B, member 1
PARP	poly ADP ribose polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGFRB	platelet-derived growth factor receptor beta
PHLDA1	pleckstrin homology-like domain, family A, member 1
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
qPCR	quantitative polymerase chain reaction
RRM	RNA recognition motif
RTK	receptor tyrosine kinase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SPRY2	sprouty homologue 2
TMA	tissue microarray
TUNEL	deoxynucleotidyl transferase doxyuridine triphosphate nick end labeling
VACD	vincristine, actinomycin D, cyclophosphamide, doxorubicin
VAIA	vincristine, actinomycin D, ifosfamide, doxorubicin

4 Introduction

4.1 Pediatric cancers

Immediately after accident injuries, cancer is the leading cause of death in children in developed countries[1]. Considering that childhood cancers represent only 2% of all cancers they are rare neoplasms[2]. The incidence rate of cancer among children under 15 years of age is about 140 per million. Despite improvements in the diagnostics and treatment of pediatric cancers that led to considerable increase in survival rates in the past several decades, the fact that these cancers are so rare still represents the major obstacle for in depth-understanding of their etiology, risk factors and biological mechanisms.

4.1.1 Epidemiology

As shown in Figure 1, there are large variations in the incidence of tumor entities among different age groups. In infants, the single most frequent neoplasm is neuroblastoma with close to one-third of all cases, while neuroblastomas, retinoblastomas, and nephroblastomas together account for about half of all malignancies. Among the 1–4-year-olds leukemias are predominant (44.9%). In age groups 5–9 and10–14 years, leukemias, lymphomas and CNS tumors represent over three quarter of all malignancies. At the same time in these age groups embryonal tumors are practically non-existent, while bone tumors are observed only then in any relevant numbers. Leukemias and CNS tumors are markedly less frequent in adolescents (15-19 years) than in the younger age groups. The number of carcinomas and germ cell tumors is considerably larger in adolescents (32.4%) than among the 10–14-year-olds (8.1%).

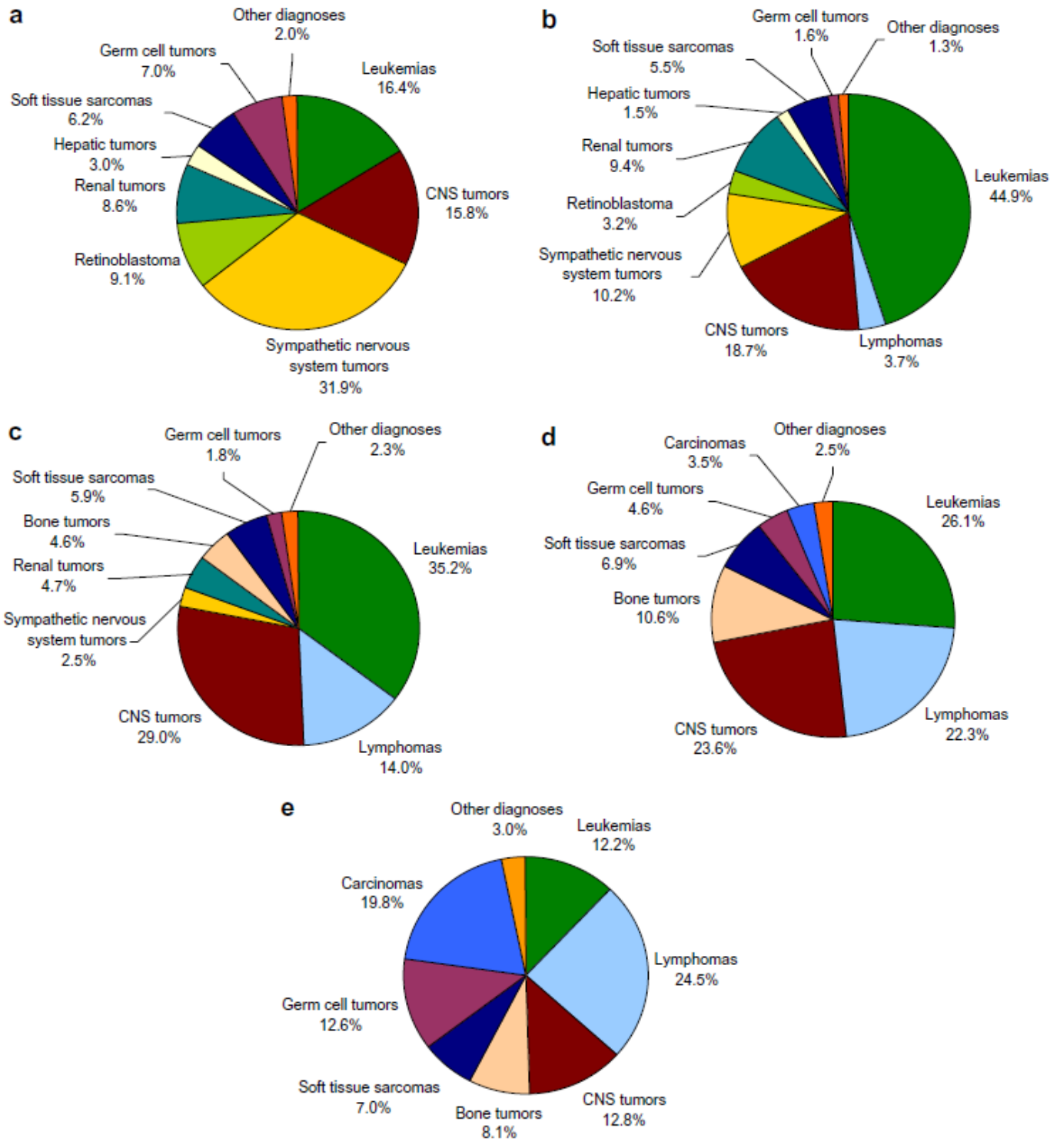

Figure 1 Relative frequencies for the main ICCC-3 diagnostic groups by age groups: (a) age <1 year; (b) age 1–4 years; (c) age 5–9 years; (d) age 10–14 years; (e) age 15–19 years (adapted from[3])

If we consider sex distribution of pediatric cancers, as shown in Figure 2 boys of any age have a higher risk of cancer than girls, with an overall sex ratio 1.2.

It is noteworthy that in the first five years of life, malignancies are about twice as frequent as among 5–14 year olds.

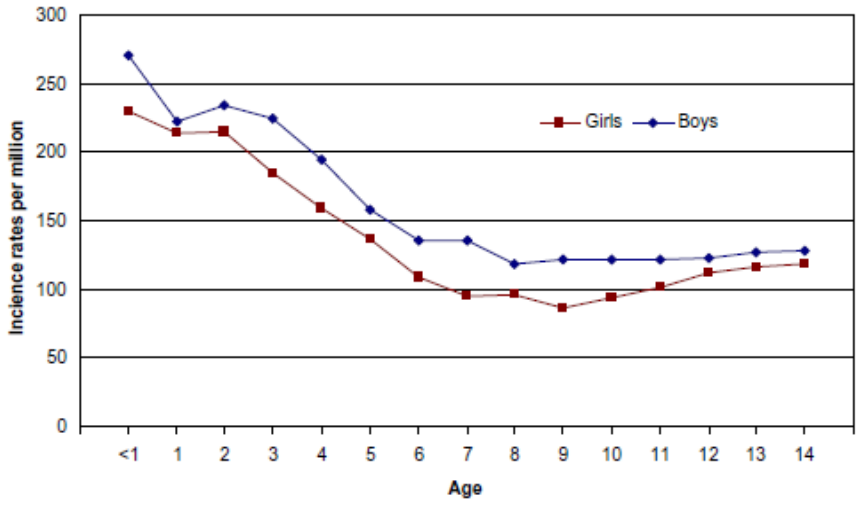

Figure 2 Age- and sex-specific incidence rates per million children for childhood cancer (age 0–14) (adapted from[3])

4.1.2 Geographical and temporal trends of incidence rates

Even though there are some geographical differences in the incidence rates, the incidence reported by 24 major cancer registries varies between 120 and 170 cases per million. However, what is of bigger concern is that increasing incidence rates were reported by a number of developed countries such as Great Britain[4], Scandinavian countries[5] and USA[6]. In Europe, an overall average annual percentage change (AAPC) of 1.1% was observed. AAPC values vary from 1.0% in the North to 1.4% in the East and from 0.9% for males to 1.4% for females. Considering the age groups, the biggest increase is seen in children younger than one year of age (AAPC: 2.1%). Out of twelve main diagnostic groups eight show a significant increase with AAPC ranging from 0.6% in leukemias to 1.8% in soft-tissue sarcomas.

4.1.3 Survival rates

Over the last few decades the survival rates for childhood cancer have improved considerably in the developed countries. The improvement in survival from the 1970s to the 1990s in the Western and Eastern Europe (East: former socialist economy countries plus Turkey without the former German Democratic Republic) is shown in Figure 3. In both Western as well as Eastern countries there was significant increase in the survival, but this improvement is less pronounced in Eastern Europe and survival rates are somewhat below the rates observed in Western Europe [7].

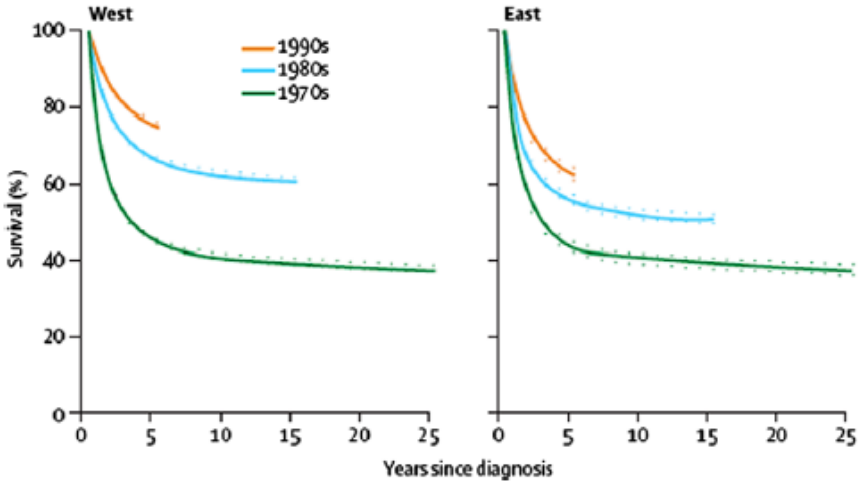

Figure 3 Survival rates by decade of diagnosis for childhood cancer (age 0–14) for East and West Europe (adapted from[3])

According to the EUROCARE data the relative risk of death was 8% lower in the 2000–2002 time period than in the years 1995–1999. In general, rates varied between 78% and 83% in the majority of countries[8]. Different data base, ACCIS (1988–1997), shows rates ranging from 62% to 77% in five European regions[9].

4.1.4 Treatment

Upon establishing a proper diagnosis, a specific therapy is assigned to each specific tumor type. This is usually a multimodal approach that includes surgical resection (in solid tumors), chemotherapy and/or radiation. The exact treatment depends not only on the tumor type but also the localization of the primary tumor as well as presence of metastases.

Table 1. Common agents used in pediatric cancer treatment and their common late side effects		
Agent	**Tumor type**	**Side effect**
Alkylating agents Ifosfamid	leukemia, sarcomas	haematuria, bladder cancer, marrow damage, leukemia, gonadal failure, fibrosis, Fanconi syndrome
Antibiotics Actinomycin D Bleomycin	sarcomas	organ dysfunction
Anthracyclines Daunorubicin Doxorubicin	leukemia, sarcomas, neuroblastoma	Heart damage, vomiting, secondary neoplasms
Antimetabolites ARA-C (HD) Methotrexan (HD) 6-mercaptopurine 6-thioguanine	lymphoma	Neurological dysfunction, Intellectual retardation, liver dysfunction, liver dysfunction
Miscellaneous Prednisone Dexamethasone Vinca alkaloids Procarbazine Cisplatin Etoposide	lymphomas, leukemia, neuroblastoma, leukemia, sarcomas	osteoporosis gonadal failure low magnesium, hearing loose, decrease glomerular filtration rate, secondary neoplasms
HD, high doses		

Cytotoxic chemotherapy is routinely used and it exploits the fact that tumor cells are fast dividing cells and hence can be distinguished from the majority of normal cells in the body. Therefore, these agents are most often targeting DNA causing impairment of either DNA replication or DNA repair and thus eliminate fast dividing cells. Other drugs, like actinomycin D target microtubuli also leading to death of fast dividing cells. Despite the enormous improvement in survival since the use of these agents, there are several problems related to this approach. First, this is a rather unspecific treatment and thus has effect on other, normal dividing cells of the body. As a consequence serious side effects including organ dysfunctions, secondary malignancies, and impairment of growth and development can be observed (Table 1). Given the fact that the patients are children, the well-being of long term survivors is essential. For this

reason there is a need for more targeted therapy approaches. Additional reason that urges the establishment of targeted therapy is the fact that the current treatment options seem to have reached a plateau of success and further intensification of the chemotherapy does not lead to increase in survival rates.

Targeted therapy approach is supposed to use the molecular differences between tumor cells and normal cells in order to specifically target just the tumor cell and spare the normal cell. There are two main approaches:

- Approach based on certain differences in the presence of molecular markers between normal and cancer cell. These cancer specific markers can then be targeted by antibodies or antibody like molecules linked to cytotoxic agents. Also, it is possible to boost immune response against cancer specific markers.

- Approach based on exploiting the differences in the dependency on some functional characteristics between normal and cancer cell. According to Benson et al. we can distinguish four dependencies[10]:

Genetic dependence refers to phenomenon also known as oncogene addiction meaning that many cancer cells are only dependent on some of the abnormalities.

Synergy dependence refers to situation in some cancer cells where mutation of one pathway renders these cells dependent on another pathway.

Lineage dependence refers to observation that cancer cells are often dependent on the same signaling pathways as their normal counterparts.

Host dependence refers to situation where either single tumor cells or the whole tumor can depend on activities coming from the host environment such as angiogenesis or growth factors produced by stromal cells.

All of the above dependencies provided possibilities for targeting that have already been successfully used to inhibit proliferation or to induce apoptosis in tumor cells while at the same time normal cell remain less affected. Therefore, it is quintessential to discover dependencies for each tumor type and thus open up a way for targeted approaches. Use of gene expression or mutational analysis as well as high throughput sequencing and other techniques has led to the

identification of novel molecular markers that can potentially be used for targeted therapy. Extensive pre-clinical validation of molecular targets will surely lead to establishment of new clinical targeted therapeutic approaches for many cancer types.

4.2 Ewing sarcoma

4.2.1 Epidemiology

Ewing sarcoma (ES) is the second most frequent bone tumor in children and adolescents. It got its name from the man who first described it, pathologist James Ewing. He believed that this tumor develops from blood vessels of the bone and hence he described it as diffuse endothelioma of the bone or later as endothelial myeloma. Today, the World Health Organization classification uses Ewing sarcoma/Primitive Neuroectodermal Tumor (ES/PNET)[11-13]. This term comprises several entities formerly known as classical ES, Askin's tumor ES of the chest wall and peripheral neuroectodermal tumor for ES with neuronal differentiation. This highly aggressive cancer affects bone and in one quarter of cases soft tissues mostly in childhood. Incidence is 1-3 per million per year which makes it a rare cancer. It has a slight male predominance and is much more frequent in Caucasians, whereas it is rarely observed in Africans[13-15]. The peak of incidence is at the age of 15 but ES continues to be diagnosed through the third decade of life. Interestingly, with the advances in molecular diagnosis of undifferentiated sarcomas, the reported incidence of ES in young adults is increasing[16-18]. Even though ES can affect any bone of the body it most commonly affect pelvis and the long bones.

4.2.2 Clinical presentation

Most often patients with ES present pain as the first tumor related symptom. This pain is in physically active patients frequently associated with athletic trauma and 10% of these patients are presenting pathologic fracture as initial symptom[19]. Further physical examination reveals palpable mass with pathologic findings in the vast majority of cases. However, in approximately 30% of the patients there are systemic symptoms such as weight loss or fever and these are often correlating with advanced stage of the disease. Reports indicate the duration of the symptoms prior to the diagnosis to be most often between 4 and 6 months[20-21]. Since ES affects bone and soft tissue, we can distinguish skeletal and extra-skeletal tumors. Approximately 80% of all ES cases are diagnosed in the first two decades of life, with 80% of these cases being skeletal ES that most commonly affects the axial skeleton (50%), with 25% of tumors occurring in the pelvis

alone[22]. In 46% of cases it arises from the diaphysis of the long bones, from these approximately 20% in femur. In primarily adult cases more than half are extraskeletal mainly affecting kidneys and uterus[13, 15, 17]. These patients usually present with vague abdominal pain. Approximately 30% of all ES patients present with detectable metastasis at diagnosis. In 35% metastasis are restricted to the lung, followed by isolated bone/bone marrow involvement, combined lung plus bone/bone marrow metastases, and only very rarely other sites. Metastases in the local lymph nodes are uncommon and in most cases are associated with disseminated disease[11, 17].

4.2.3 Diagnosis, imaging and staging

If there is a suspected tumor the first step in the assessment phase is imaging (Figure 4). In the case of assumed osseous lesions, initial imaging is a radiograph in 2 plains. Radiographic signs of ES are tumor osteolysis accompanied by periosteal reaction such as onion skin pattern, calcified spiculae or Codman triangle[23]. The exact extent of the local tumor must be determined by computed tomography (CT) or magnetic resonance imaging (MRI). Even though in assessing the osseous lesion CT is superior to MRI, MRI can more accurately define the intra medullary and soft tissue extent of ES as well as anatomic relations to adjacent vessel/nerve bundles, which is crucial in planning biopsy and local therapy. All local imaging techniques should display the full compartment including adjacent joints to assess skip metastases. In order to avoid hemorrhage and edema artefacts, local imaging must be completed before biopsy[24]. Nevertheless, the definitive diagnosis of malignant bone tumors requires biopsy proof. To achieve this core or fine-needle biopsy needs to be performed. ES often has extensive necrosis and therefore it is necessary to make sure that sufficient material is harvested for diagnosis, including subsequent immunohistochemical and molecular analyses. Hence, usually several cores are necessary, with frozen sections verifying representative sampling. Despite the fact that these methods are intended to expedite diagnosis, uncertain results even in experienced centers may actually delay diagnosis[25]. Therefore, an open biopsy is still considered the standard approach in many specialized sarcoma centers. Biopsies need to be carefully planned following clinical examination and complete local imaging, since any biopsy tract is considered contaminated and should be marked indelibly (e.g., with a tattoo or incision) so that it can be excised in definitive tumor resection and/or included into the radiation field of definitive radiation. That is exactly why biopsy should be done by the same multidisciplinary oncology team responsible for final local treatment.

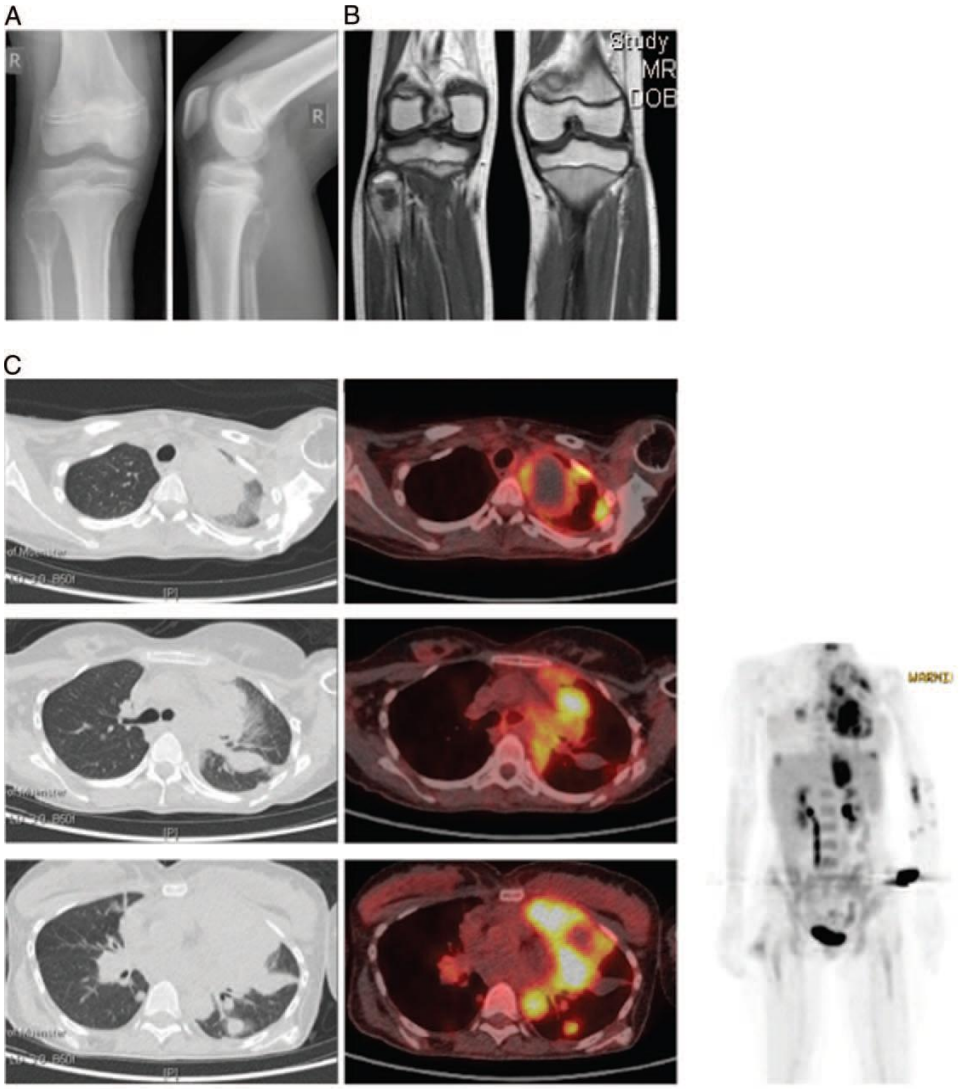

Figure 4 ES diagnostic imaging and histology. (A) Conventional radiograph in 2 planes of right fibula ES. (B) Corresponding MRI (T1-weighted image with gadolinium contrast). (C) PET-CT of advanced ES with pulmonary metastases. Left panels, chest CT; right panel, whole-body FDG-PET; middle panels, fusion images with FDG tracer uptake by viable metabolically active tumor fractions, color-coded (adapted from[26])

Upon pathological diagnosis of ES, further staging is focused on assessment of metastases, which are detected in approximately 25% of patients. The usual staging procedures are chest CT scan, 99m-technetium whole-body bone scintigraphy, and bone marrow aspirate and biopsy. Even today bone scintigraphy remains an indispensible standard for the detection of bone metastases. Recently it is supplemented with metabolic[18F] fluoride oxyglucose positron emission tomography (FDG-PET), increasingly performed as fusion PET-CT together with a diagnostic chest CT scan[27]. First prospective studies show superiority of FDG-PET in assessing bone/bone

marrow and soft tissue involvement compared with conventional staging, whereas PET-CT or CT is more reliable than FDG-PET in depicting lungmetastases[28-31]. With this improvement it is possible to expect that FDG-PET findings may lead to changing the stage and consequently the treatment group in some patients. Every one of these techniques has its limitations in terms of sensitivity and specificity. With improvements these techniques are evolving so it is necessary to establish clear criteria for clinical trials. However, even though the sensitivity of diagnostic imaging has been improved in the last decades, the fraction of patients with detectable metastases at diagnosis has remained constant at 20–25%

4.2.4 Histology

Morphologically ES cells are characterized by a high nuclear to cytoplasm ratio and they exhibit faintly eosinophilic cytoplasm containing glycogen appearing granules that stain positive for periodic acid–Schiff (PAS). Mitotic activity is low, cytoplasmic borders are indistinct and nuclei are round with evenly distributed chromatin [32]. Most tumors express surface antigen CD99 as well as vimentin.

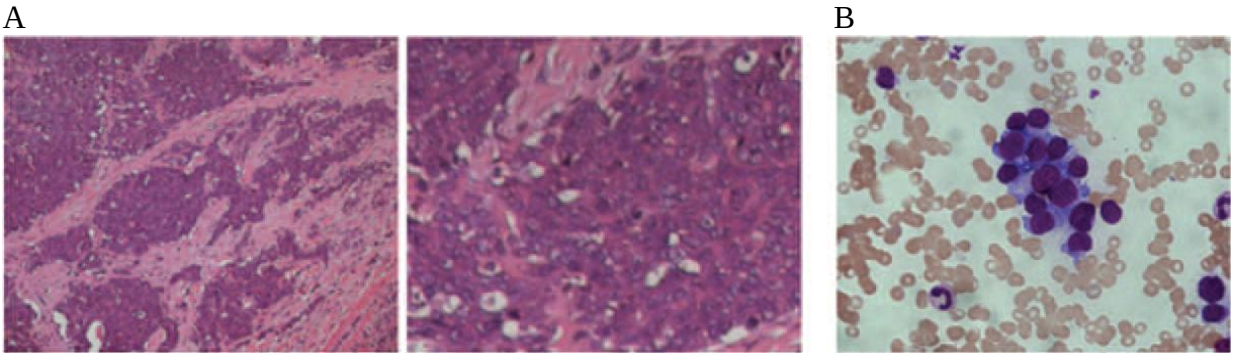

Figure 5 ES histology (A) H&E staining, Left panel, 20x magnification; right panel, 40x magnification. (B) ES bone marrow metastasis (smear from bone marrow aspirate) (adapted from[26])

ES, together with neuroblastoma, alveolar rhabdomyosarcoma and lymphoblastic lymphoma belongs to a group of small blue round cell tumors that exhibit poorly differentiated cell phenotype (Figure 5). Since histology and in part immunophenotype of ES overlaps with these other small round blue cell tumors, a complex immunohistochemical approach with analysis of multiple markers is necessary in order to establish the correct diagnosis. ES shares expression of CD99 with lymphoblastic lymphomas, but the difference comes from the expression of CD45 in lymphoblastic lymphomas that is absent in ES. Alveolar rhabdomyosarcoma might also express

CD99 but it expresses desmin, myogenin and MyoD1 that are not expressed in ES. Expression of neural specific enolase (NSE) and S-100 is common to both neuroblastomas as well as ES. However, neuroblastomas are vimentin-negative and neurofilament-positive whereas Ewing sarcomas are not[33]. Poorly differentiated small synovial sarcoma might express CD99 but lacks other histological markers which makes it difficult to set histology based differential diagnosis. If the tumor shows neuroectodermal features it is considered as PNET.

All these similarities in the expression of antigen markers along with poorly differentiated morphology of this family of tumors made the diagnostic very difficult. However, ES like some other sarcomas has specific chromosomal rearrangements that can be used as biomarkers. Discovery of these genetic events provided unambiguous diagnosis based on molecular techniques like fluorescence *in situ* hybridization or polymerase chain reaction.

4.2.5 Prognostic factors

The single most important prognostic factor is the stage of the disease which basically comes down to one question, presence or absence of metastases at diagnosis. Also, there is different prognostic relevance depending on the site of metastases. Thus, patients with pulmonary metastases have better prognosis than those with bone metastases[16, 34-35]. In addition, detection of tumor cells in the bone marrow by detecting fusion transcripts using RT-PCR may have prognostic value[36-37].

Apart from metastases, the main prognostic factors in ES are tumor volume and the site of the primary tumor, chemotherapy response and the age of the patient[16-17, 34-35]. Tumor necrosis induced by chemotherapy has been shown in several studies to be the most important indicator of event free survival in ES patients with surgical treatment after induction chemotherapy[17, 38-39].

Among cytogenetic aberrations with prognostic significance, gain of 1q and loss of 16q are reported to have inverse correlation to outcome[40-41]. Also, presence of mutations/alterations in p53 and p16 are correlated with worse prognosis[42].

4.3 Biology of Ewing sarcoma

4.3.1 Chimeric fusion protein

ES represents morphologically heterogeneous tumors but they all have one common feature which is nonrandom chromosomal translocation involving the EWS gene and one of the members of the ETS family of transcription factors[43]. Approximately 85% of ES tumors have the t(11;22)(q24;q12) chromosomal abnormality[44]. This translocation leads to in frame fusion of the EWSR1 (also known as EWS) gene on chromosome 22 to the FLI1gene on chromosome 11 and encodes the EWS/FLI1 fusion protein[45] (Figure 6). EWS/FLI1 contains the amino-terminus of EWS fused, in frame, to the carboxyl-terminus of FLI1transcription factor[46]. In ES cases lacking EWS/FLI1, there are alternative translocations present that fuse EWS to other ETS family transcription factors, like ERG (5% of cases), ETV1, ETV4, FEV and others[47-52], which all most likely mimic EWS/FLI1[52-55]. Moreover, the presence of the fusion enabled easy differential diagnosis by performing PCR and thus distinguishing ES from other histo/morphologically similar tumors such as neuroblastomas, rhabdomyosarcomas and giant cell tumors[56].

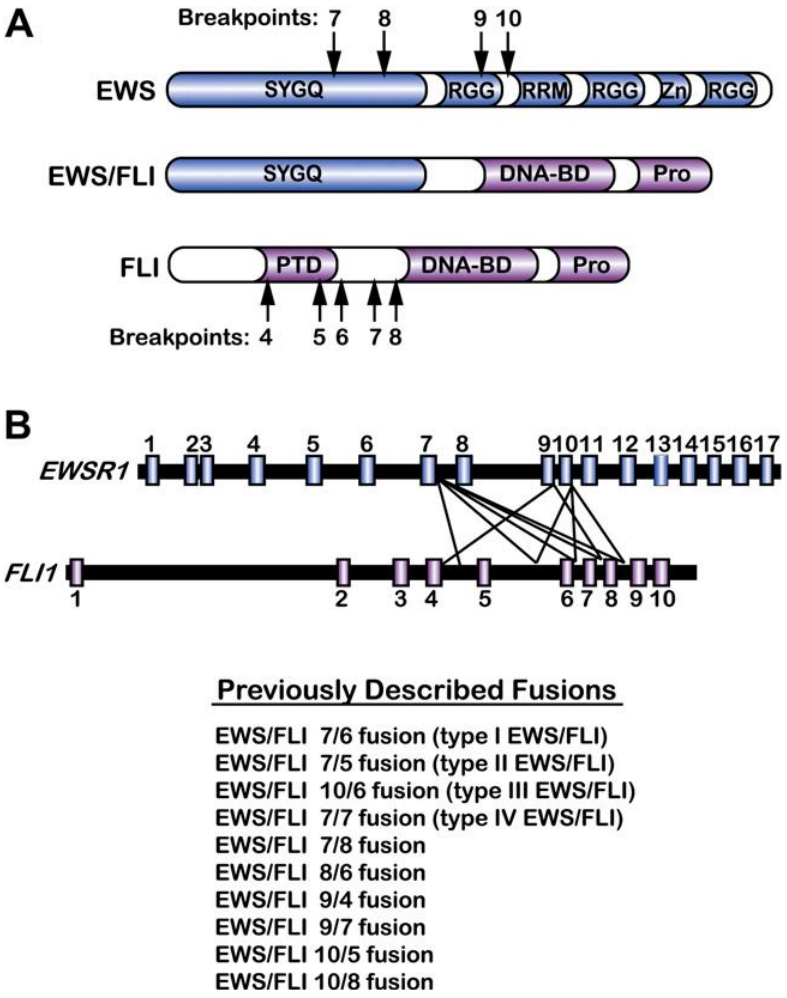

Figure 6 (A) Domain structures of wild-type EWS, wild-type FLI and the EWS/FLI fusion proteins. SYGQ: serine tyrosine-glycine glutamine rich transactivation region; RGG: arginine-glycineglycine rich regions; RRM: RNA recognition motif; Zn: putative zinc finger; PTD: pointed domain; DNA-BD: DNA binding domain; Pro: proline-rich activation domain. Arrows indicate breakpoints in wild-type EWS and FLI included in commonly observed subtypes of the EWS/FLI fusion protein. (B) Genomic structures of the EWSR1 and FLI1 genes. Breakpoints in the EWSR1 and FLI1 genes occur in many introns. Following splicing, the exons join together to generate various subtypes of EWS/FLI. Some of the previously described EWS/FLI fusion subtypes are depicted here. (adapted from[57])

Apart from encoding for EWS/FLI1 fusion protein, the chromosomal rearrangement t(11;22)(q24;q12) has two additional consequences. First, due to this translocation EWS/FLI1 is under the control of EWSR1 promoter and hence it is constitutively expressed in tumor cells. Secondly, there is haploinsufficiency for these two genes. When it comes to FLI1 copy it probably has no major consequence since FLI1 is only expressed at very low levels in ES cells. However, the consequence of disrupting one copy of EWSR1 is not understood at the time.

4.3.2 Different EWS/FLI1 fusion types

As a result of different genomic breakpoints usage in the EWSR1 and FLI1 genes there will be generation of different, highly related isoforms of EWS/FLI1. All breakpoints occur within introns of these two genes and the final fusion is also within introns[58-60]. Upon splicing of the transcribed RNA, the mRNA incorporates 5' exons from EWSR1 fused to 3' exons derived from FLI1[45, 58-59]. These splicing variants are named according to the frequency of a certain fusion type. The most frequent so called type I fusion is a fusion between exon 7 of EWSR1 and exon 5 of FLI1[45]. For the first 4 most frequent rearrangements the same nomenclature applies, thus they are referred to as Type I, Type II, Type III and Type IV. For the other much less frequent fusion subtypes there is no strict nomenclature. The influence of these minor differences has been extensively investigated as a prognostic factor. It was at first suggested that it has a prognostic value[61]. Some earlier studies implied that patients with Type I fusion have better relapse free survival but recent reevaluation of this claim has been done in a large cohort of patients. In this study it has been excluded that fusion type has any impact on disease progression or survival[37, 62].

4.3.3 The EWS Protein

The EWS protein is a member of TET family which also includes liposarcoma fusion protein TLS/FUS and the human TATA binding protein-associated factor (hTAFII68)[63-64]. These are RNA-binding proteins containing a transcriptional activation domain(s) in the N-terminus and an RNA recognition motif in their C-terminus[65-66]. Since they lack a secondary structure this suggests a probable involvement as cofactor. However, structure of TET proteins doesn't imply that they function as classical transcription factors or co-factors, given the fact that they contain RNA recognition motifs and have been shown to bind RNA as well as both single-stranded and double-stranded DNA[64, 66-68]. Interestingly, expression of TET proteins is associated with a number of solid tumors such as myxoid chondrosarcoma (EWSR1-NR4A3, TAF15-NR4A3, TCF12-NR4A3, TFG-NR4A3), angiomatoid fibrous histiocytoma (FUS-ATF1, EWSR1-ATF1, EWSR1-CREB1) where their N-terminal part is being involved in chromosomal translocations with various transcription factors[69]. The EWS protein is expressed in all tissues and can be considered housekeeping gene. It's mainly located in the nucleus, even though it is present in smaller amounts at the cell membrane and the cytoplasm[70-71]. It is expressed ubiquitously with similar levels throughout the cell cycle. The transcriptional potency of the N-terminal domain of EWS that is observed in different tumorigenic fusion proteins suggests that it may play a role as a

transcription factor. Also, it has been shown to play a role in splicing, in RNA processing, translation inhibition and in cytoskeleton stabilization[72-76].

4.3.4 The Fli1 Protein

This ETS transcription family consists of approximately 30 genes divided in distinct subfamilies. Among others they include *FLI1*, *ERG*, *ETV1*, *E1AF*, *FEV* and *ZSG*. In cooperation with other transcription factors and cofactors the ETS transcription factor family of proteins is involved in a variety of mammalian developmental processes at the cellular, tissue and organ levels[77]. They are implicated in cellular proliferation, migration, differentiation, apoptosis and cell-cell interactions[78-79]. They are important during embryogenesis and their expression in adults is strictly regulated[80]. All ETS protein members share a highly conserved DNA binding domain through which they bind to sequences containing a GGAA 'core' motif surrounded by bases that provide affinity and specificity to the interaction[81-82]. FLI1 is transcription factor with very restricted expression, especially during development, preferentially in hematopoietic and endothelial cells, and in the mesenchyme derived from neural crest cells[83]. In adult tissues FLI1 remains expressed almost only in hematopoietic tissues. Fli1 as well as other ETS proteins are known to act both as activator as well as repressor of transcription having as targets oncogenes, tumor suppressor genes and genes related to apoptosis, differentiation, angiogenesis and invasion[78, 81, 84].

4.3.5 Cell of origin

To understand ES it is crucial to address the issue of the cell of origin. Even though a substantial amount of work has been done on identification and characterization of the cell of origin there is still no clear answer to this question. There exist two hypotheses:

- Mesenchymal stem cell as the cell of origin for ES
- Neural crest stem cell as the cell of origin for ES.

There are several observations going in favor of neural crest stem cell of origin[85-87]. First it was found that ES expressed cell surface antigens associated with the neuroectodermal lineage[86]. Both ES and peripheral primitive neuroectodermal tumor have the same t(11;22)(q24;q12) rearrangement suggesting they represent the same tumor with differences in extent of neural

differentiation[44, 88-89]. Finally, a gene expression profiling study showed that genes expressed in neural tissues or during neuronal differentiation are highly expressed in ES[87].

Regarding mesenchymal stem cells as the cell of origin, in the past few years there is a slight prevalence in favor of this theory that has been collaborated by growing amount of evidence[90-94]. Upon silencing of EWS/FLI1 in ES cell lines they displayed a mesenchymal stem cell (MSC) gene expression profile, and they were capable to differentiate along both osteogenic and adipogenic lineages[94]. Also, human MSCs provide an appropriate cellular context for EWS/FLI expression. Human MSCs retain the ability to propagate in the presence of the fusion protein unlike other normal human cell types when transfected with EWS/FLI1[93].

However, it is possible that these two theories are not mutually exclusive. One new idea arose that is encompassing both of these two cell types, suggesting that ES may arise from a neural crest stem cell exhibiting mesenchymal features or from a mesenchymal stem cell that is neural derived[95-96].

Regardless of which hypothesis is correct it is necessary to emphasize the importance of the appropriate cellular context for the study of EWS/FLI1 and its function. Despite the fact that EWS/FLI1 has transforming ability when transduced in NIH3T3 cells, expression of EWS/FLI1 results in growth arrest in a variety of different cell types including primary human fibroblasts, mouse embryonic fibroblasts, immortalized rat fibroblasts, neural crest progenitor cells and rhabdomyosarcoma cells[97-98]. Further support for the importance of cellular context comes from the fact that when EWS/FLI1 is expressed in NIH3T3 cells it activates a different gene expression pattern from the one observed in ES cells[53]. Even putative cells of origin, namely human mesenchymal stem cells, fail to form tumors when expressing EWS/FLI1 and injected in immunodeficient mice[93]. Altogether, it is clear that for the tumor development not only EWS/FLI1 expression is necessary but also the appropriate cellular background with additional secondary hits. This is exactly the reason why there is still no animal model for ES since, apart from the issue of the right cell of origin, we don't know what are the secondary hits necessary for ES development.

4.3.6 EWS/FLI transcriptional regulation

EWS-FLI1 acts as aberrant transcription factor by altering expression of target genes. Since many of its target genes contribute to tumorigenesis, substantial amount of work has been carried out to

elucidate both mechanisms of transcriptional regulation by EWS/FLI1 as well as to identify the crucial target genes for the biology of ES. Even though first studies classified EWS/FLI as a strong transcriptional activator[46, 65, 99-100], later it was shown that it can also function as a transcriptional repressor[101-102]. Further expression profiling of ES cell lines confirmed the repressive capacity of EWS/FLI1[103-104]. Interestingly, among more than 1000 EWS/FLI-regulated genes in A673 cells, it appeared that down regulated targets comprised up to 80% and therefore the majority of the deregulated genes. However, more recent comprehensive transcriptional analyses comparing multiple ES cell lines and tumor samples discovered a more equal representation of EWS/FLI induced and repressed genes[105-107]. Several studies demonstrate the importance of target gene repression for the fully malignant phenotype and imply that this may be a critical EWS/FLI1 mediated activity[101, 103].

EWS/FLI1, like other ETS family members, is binding to DNA sequences containing a GGAA core motif surrounded by additional bases necessary for high affinity and specificity of the interaction[81-82]. Using in vitro binding site selection approaches it has been shown that both wild type FLI1 as well as EWS/FLI1 preferentially bind to ACCGGAAGTG sequence with high affinity[81, 108]. The binding of EWS/FLI1 to this sequence was confirmed also in vivo using ChIP on chip approach[109]. However, in the case of EWS/FLI1 up regulated target genes, GGAA-containing microsatellite sequences have been identified as EWS/FLI-binding sites and this has been confirmed by ChIP-sequencing as well[110]. The identification of microsatellites as cancer-relevant EWS/FLI-binding sites suggests that such elements have important roles in the development of ES despite the fact that microsatellite sequences are usually described as ‘junk DNA’ without biologic function[111]. The exact mechanism of how EWS/FLI1 is binding to these microsatellites is not fully elucidated but it might hold true for other ETS family members[112].

The exact mechanism of EWS/FLI1 transcriptional activation and repression is not fully elucidated. Protein–protein interactions and post-translational modifications are probably involved in both activating and repressing functions of EWS/FLI1. In ES cell lines EWS/FLI1 has been shown to be phosphorylated, and in a heterologous system phosphorylation modulates DNA binding and transcriptional activity, suggesting that phosphorylation may be important for the function of EWS/FLI1[113-114]. Other posttranslational modifications of EWS/FLI1, such as O-GlcNAcylation might be involved in the regulation of EWS/FLI1 transcriptional activity[115].

EWS/FLI1 interacts with proteins involved in the transcriptional process like RNA polymerase II subunit RPB7[72]. Apart from the basal machinery, EWS/FLI interacts with other transcriptionally relevant proteins, such as RNA helicase A[116]. Inhibition of this association with small molecules reduced tumor growth in xenograft mouse models, suggesting that this interaction is biologically relevant[117]. Using reporter assays it was shown that the RNA helicase A-EWS/FLI1 interaction increases transcriptional activity of EWS/ FLI1. However, the presence of RNA helicase A at the promoters of EWS/FLI1 repressed target genes doesn't go along with these findings[116].

The mechanism of transcriptional repression by EWS/FLI1 is less clear. One explanation might be that interaction between EWS/FLI1 and p300 is inhibiting the histone acetyl-transferase activity of p300, thus blocking its transcriptional activation and resulting in transcriptional repression[102]. However, there is more evidence for an indirect repressive function of EWS/FLI1 by up regulating expression of transcriptional repressors, such as NKX2.2, or co-repressors like NROB1[93, 118-119].

Genomic approaches linking RNAi technologies with microarray analysis have identified thousands of EWS/FLI1 deregulated genes in ES cell lines[103-104, 106-107]. However, only a hand full of genes have been validated as direct target genes of EWS-FLI1: hsRPB7[72], UPP1[120], tenascin-C[121], Id2[122], p21WAF1/CIP1[123], PTPL1[124], phospholipase D2[125], TGFBR2[101], IGFBP3[126], MK-STYX[127], TERT[128], GLI1[126], NR0B1[129], caveolin-1[130], EZH2[93] and Aurora A and B[131]. Many more genes have been described but not confirmed to be direct targets, despite the fact they are involved in signaling pathways and gene regulation networks initiated by EWS-FLI1. These indirect target genes or yet to be validated target genes include: EAT-2[132], MFNG[133], mE2-C[134], PIM3[135], IGF1/IGF1R[136], c-Myc and p57KIP2[137], MAPT, PP1R1A, NEK2 andcyclin D1[138], Skp2[139], CD99[140], zyxin[141], cholecystokinin[142], VEGF-A[143], NKX2.2[104], NOTCH-p53[144], thrombospondins1 and 2[145] and TOPK[146]. Although not all of these genes are actively involved in the transformation process, some of them have been shown to be crucial for oncogenesis (NKX2.2, NR0B1and GLI1)[104, 107, 119, 147-149]. All three of these proteins have a role in transcriptional regulation. NKX2.2 is transcription factor that is a critical direct EWS-FLI1 target gene and required for ES oncogenic transformation[104]. Interestingly, the NKX2.2 transcriptional signature was found to consist exclusively of down-regulated genes that overlap with the EWSFLI1 repressed genes in ES[119]. This implies that at least to some extent EWS/FLI1

repressive function can be attributed to other transcriptional repressors that are directly upregulated by EWS/FLI1.

In contrast to target genes involved in transcriptional regulation, other EWS/FLI1 target genes can be connected to hallmarks necessary for tumor formation and progression[150]. For example, expression of hTERT allows for tumor cell immortalization and expression of vascular endothelial growth factor allows vasculogenesis. Exactly these two genes are expressed in many ES tumors and cell lines[151-155]. Both of these genes have been shown to be induced by EWS/FLI in heterologous cellular backgrounds[152-153].

The role of many of EWS/FLI1 target genes has been elucidated by now. Up regulation of target genes like IGF1, MYC and NKX2.2, promotes cell proliferation and survival. At the same time inhibition of other target genes like p21, p57kip, TGFBR2 and IGFBP3 leads to escape from growth inhibition, senescence and apoptosis. Taken together, EWS/FLI1 as a hallmark of ES through transcriptional regulation of many target genes drives tumor development, maintenance and progression.

4.4 Treatment of Ewing sarcoma

4.4.1 Multimodal treatment

Before chemotherapy has been introduced, survival of ES patients was less than 10%, even though it was well known that this tumor type is very sensitive to radiotherapy. Today, in patients with localized disease by applying modern multimodal therapeutic regimens including induction chemotherapy and local control with surgery, radiotherapy or a combination of both modalities, cure rates of approximately 70% can be achieved. However, for patients with metastasis at diagnosis the prognosis is still very bad, indicating the limitations of current therapeutic approaches. Intensity of chemotherapy is of significant importance.

Ewing Sarcoma	
First-line: Neoadjuvant/ Adjuvant or Primary Metastatic Disease	Second-line: Relapsed or Refractory Metastatic Disease
Vincristine, doxorubicin, and cyclophosphamide alternating with ifosfamide and etoposide (VAC/IE)	Cyclophosphamide and topotecan
Vincristine, ifosfamide, doxorubicin, and etoposide (VIDE)	Temozolomide and irinotecan
Vincristine, ifosfamide, and doxorubicin (VIA)	Ifosfamide and etoposide
	Ifosfamide, carboplatin, and etoposide
	Docetaxel and gemcitabine

Table 2 Chemotherapy in ES (adapted from[156])

Starting with single-agent chemotherapy in the early 1960s, modern multiagent chemotherapeutic regimens have been developed[157](Table 2). Ever since, a huge effort was undertaken to find optimal treatment strategies: actinomycin D, cyclophosphamide, and vincristine were introduced in the 1970s[158]; neoadjuvant chemotherapy concepts were first introduced by Rosen et al and consecutively developed by other groups[20, 159-161]. The benefit of additional anthracyclines was shown in the 1980s[17, 20, 162]. Since 1990 the standard neoadjuvant/adjuvant chemotherapy backbone consists of vincristine, actinomycin D, cyclophosphamide, and doxorubicin (VACD or VACA)[163]. In the meantime, a number of studies have been initiated with the goal to improve this standard protocol. In study INT-0091 conducted by the Pediatric Oncology Group–Children's Cancer Group (POG-CCG),36518 ES patients were randomized to chemotherapy with VACD either alone or alternating with ifosfamide and etoposide (VACD-IE). Compared with VACD alone, the alternating VACD-IE regimen significantly improved 5-year event free survival (69% *v* 54%, *P*=0.005) and 5-year overall survival (72% *v* 61%, P=0.01). The European Inter-group Cooperative ES Study-92 (EICESS-92) compared the activity of VACA with a similar regimen in which cyclophosphamide was replaced with ifosfamide (VAIA) in 155 standard-risk patients[164]. The 3-year event free survival rates for VACA was 73% and for VAIA was 74%, thereby indicating that these two regimens have similar activity in ES. The only advantage of VAIA was that it induced less hematologic toxicity. The EICESS-92 study, in 492 high-risk patients,

compared VAIA with VAIA plus etoposide (EVAIA)[164]. However, this comparison showed only a non-significant 17% improvement in event free survival and a 15% improvement in overall survival among patients treated with EVAIA. Currently, the most widely used regimens contain vincristine, doxorubicin, and cyclophosphamide, with ifosfamide and etoposide with or without actinomycin D (Table 2).

There are ongoing clinical trials that are supposed to establish whether the addition of high dose chemotherapy regimens followed by autologous haematopoietic stem cell rescue has some advantage. The current standard is, following biopsy-proven diagnosis, initiating four or five drug combination chemotherapy schema that isifosfamide and doxorubicin based. This treatment is continued following local treatment for a total duration of 10−12 months. The rationale for primary chemotherapy is to induce shrinkage of the primary tumor and thus facilitate local control, especially limb salvage surgery, which is easier to perform once an initial bulky soft-tissue mass has disappeared under intense primary combination chemotherapy. With respect to the modality of local control, there is good evidence from various groups regarding the better control with surgical or combined surgical and radiotherapy modalities compared with definitive radiotherapy that bears a higher risk of local recurrence and secondary radiotherapy induced malignancies. However, this might be a biased result given the fact that definitive radiotherapy is usually used in difficult to resect large tumors that are not located in limbs but more often in pelvis and spine, sites difficult to resect which are associated with worse prognosis[165]. As a result of current intense combination chemotherapy regimens and careful interdisciplinary local therapy planning, with a preference for surgery or combined modality local control, long-term disease-free survival in tumors of the extremities is approximately 80%[166]. However, pelvis remains the most difficult primary site. In this case patients usually already have a large mass at diagnosis and this is followed by further difficulties regarding appropriate surgery and radiation that all together contribute to worse prognosis[164, 167-168].

Nevertheless, the single most important adverse prognostic factor is presence of metastases at diagnosis. If the metastases are in the lungs, using lung irradiation appears to improve the outcome. In patients with multiple osseous metastases at diagnosis there is direct correlation between long term survival and the extent of disease within the skeletal system and expected long term survival is less than 10%[34, 169-170].

4.4.2 Targeted treatment

The aim of targeted therapy is to specifically eliminate tumor cells while at the same time normal cells remain less affected. ES cells, due to their unique properties, present quite good candidates for targeted therapy. The presence of EWS/FLI1 fusion protein only in the tumor cell distinguishes it from normal cells. At the same time, EWS/FLI1 alters expression of many target genes creating the fully malignant phenotype of the tumor cell leading to 'oncogene addiction', makes tumor cells dependent on its presence. Since EWS/FLI1 has these properties only in the permissive background, this implies dependency of the tumor cell on synergistic signaling pathways that cooperate in EWS/FLI1 permissive cellular background.

4.4.2.1 Targeting EWS/FLI1

Targeting oncogenic fusion proteins is quite promising since they represent unique molecular targets for tumor cell specific therapeutic development. Imatinib (Gleevec) as targeted inhibitor of the BCR-ABL fusion protein kinase provides proof of principle for this strategy and it completely revolutionized chronic myelogenous leukemia (CML) treatment. However, unlike kinases, the transcription factor fusion proteins that are present in sarcomas remain un-druggable.

Reducing EWS-FLI1 levels by antisense oligodeoxynucleotides[171-173], antisense RNA expressed from a vector[171, 174-175], and small interfering RNA (siRNA) delivered via nanoparticles[173], all inhibit the proliferation ES cell lines and xenografted tumors. However, the challenge of pharmacologic delivery still remains the main obstacle for the clinical translation of these macromolecule based strategies[176].

For pharmacological reasons small-molecule inhibitors of EWS-FLI1 function may be more favorable. However, since EWS/FLI1 lacks an intrinsic enzymatic activity finding a small molecule inhibitor is difficult. Cytosine arabinoside (ARA-C) has been identified in a small molecule screen as potential modulator of EWS-FLI1, and it was suggested to decrease EWS/FLI1 protein expression[177]. Being a promising candidate with longstanding experience for treatment of childhood leukemia, ARA-C has been rapidly translated into a phase II clinical trial. However, intermediate doses of ARA-C failed as a single agent in relapsed and refractory ES patients, having only minimal activity but profound toxicities[178].

Recent high-throughput screening of more than 50 000 compounds identified mithramycin as specific inhibitor of EWS/FLI1 activity[179]. The compound exhibited strong antiproliferative

effect in ES cell lines in low nM concentrations and also antitumor activity in vivo. Even though the mechanism of action is not known yet, Grohar et al. speculate that mithramycin is directly inhibiting EWS/FLI1 activity[179].

A potentially useful strategy to target EWS-FLI1 is disruption of key EWS-FLI1 protein-protein interactions since protein complexes have been suggested to regulate other ets-family proteins[180]. Even though disruption of protein-protein interactions is very challenging it seems that the therapeutic disruption of EWS-FLI1 transcriptional complexes might be feasible. RNA helicase A (RHA) has been identified as a protein that specifically binds EWS/FLI1, enhances EWSFLI1 modulated transcription and cooperates in EWS-FLI1 oncogenic transformation[116]. Although the exact nature of RHA requirement for EWS-FLI1 function remains to be determined, small molecule inhibitors of RHA–EWS/FLI1 protein-protein interaction have been identified[117]. YK-4-279 is small molecule that inhibits tumor growth in ES xenograft model. These data provide a first proof of principle that disruption of the interaction of a cancer specific oncogenic transcription factor with its normal cellular binding partners provides a strategy for tumor cell-specific therapeutic development.

4.4.2.2 Targeting Growth Factor signaling

Growth factor signaling usually goes via cell surface receptors whose activity is ligand dependent. These receptors possess tyrosine kinase activity in their intracellular domains and when activated they transfer this signal to downstream signaling cascade of kinases. In many cancers RTKs are dysregulated so that they are constitutively active thus presenting very potent oncoproteins involved in development and progression of cancer. For this reason RTKs, their ligands and downstream signaling molecules with kinase activity are a focus for targeted drug development.

4.4.2.3 Targeting the insulin-like growth factor-1 receptor (IGF1R)

In solid cancers, the IGF1R gene is often amplified while at the same time ligand over expression is frequently observed, altogether resulting in enhanced signaling[181-182]. The contribution of the insulin-like growth factor (IGF) pathway to pathogenesis of ES has been extensively discussed[183-184]. Binding of IGF-I to the insulin-like growth factor I receptor (IGF-IR) leads to activation of PI3K and MAPK pathways promoting proliferation[185] (Figure 7). A link between IGF-I and ES has been implied also by epidemiological studies. The rising IGF-I levels in puberty coincide

with incidence of ES in this age, since ES most frequently occurs in the second decade of life[186-188]. Therefore, it appears that IGF1R signaling is crucial for ES development.

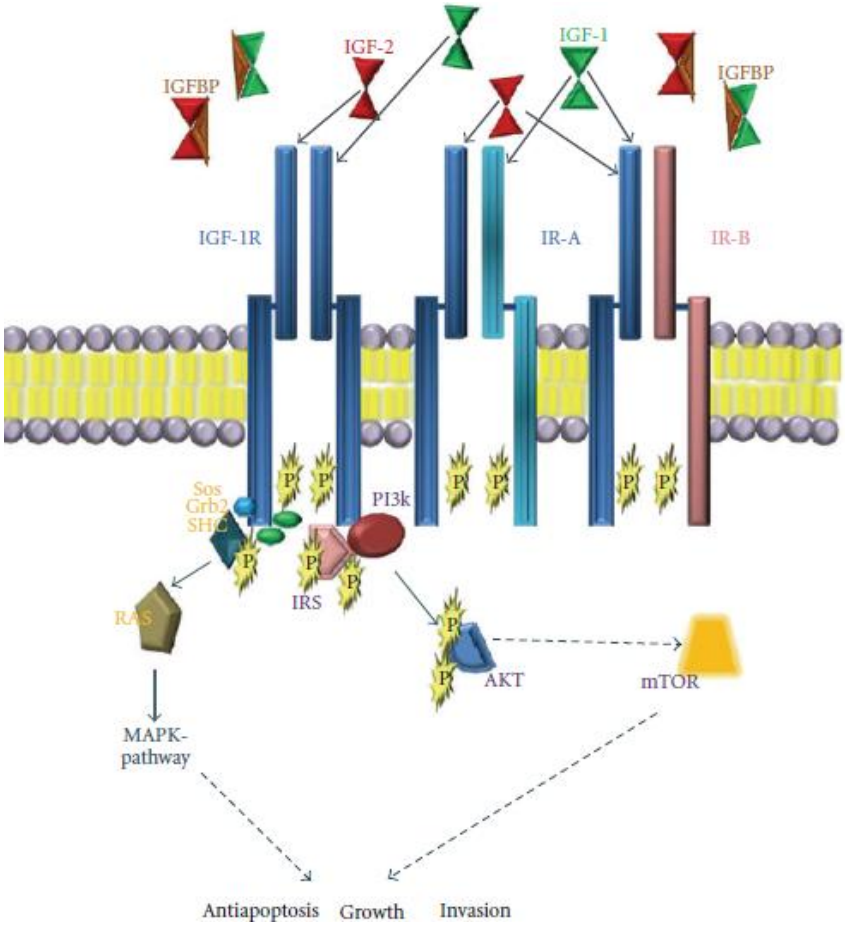

Figure 7 IGF1R signaling activation upon binding of ligands leads to activation of MAPK and PI3K pathways. Solid lines indicate direct interaction (adapted from[189])

Clinical efficacy of IGF1R blockage has a wide molecular basis in ES[180, 186]. In patients with metastatic disease and low IGF-I levels and high IGFBP3:IGF-I ratios a trend towards improved survival has been observed[187]. An IGF1/IGF1R autocrine circuit exists, where EWS/FLI1 up regulates IGF1 ligand as a direct target gene[190]. Molecular studies have shown that EWS/FLI1 through aberrant transcription represses negative regulator of IGF1, insulin-like growth factor binding protein 3 (IGFBP3) thus increasing the bioavailability of IGF1[103]. EWS/FLI1 also indirectly diminishes level of IGFBP3, in NIH3T3 cells and embryonic stem cells, through direct repression of transforming growth factor beta receptor type II (TGFβRII) expression. TGFβ was shown to induce IGFBP3[101, 191-192]. Also, for EWS-FLI1 transformation of fibroblasts a functional IGF1R signaling axis is necessary[193]. Blockage of IGF1R signaling in ES cells in vitro increases

sensitivity to conventional chemotherapeutics, leading to impaired tumor growth, metastasis, and angiogenesis in vivo[194-197].

All these findings are in good agreement with the basic presumption for targeted therapy and which in this case is existence of an 'addiction' to IGF1R signaling. Therefore, blocking IGF1R signaling should induce apoptosis in ES cells and spare the normal cells thus opening the possibility of tumor cell specific targeted approach. Interestingly, a similar IGF1R addiction has been observed for other fusion protein bearing childhood sarcomas, namely rhabdomyosarcoma and congenital fibrosarcoma[198-199].

Induction of apoptosis through inhibition of IGF-IR with a monoclonal antibody was observed as early as 1990[136, 200-201]. Based on all of the above, several IGF-IR antibodies have been developed and are being tested in phase I/II studies in ES patients. Human monoclonal IGF1R antibody AMG479 (ganitumab), in a phase I study, in patients with advanced solid tumors or non-Hodgkin lymphoma (NHL), induced complete response of more than 28 months in one ES patient[202]. In another study, the fully human monoclonal antibody R1507 (robatumumab) was well tolerated and showed activity in patients with advanced solid tumors, with good response in two ES patients[203]. And in a phase I study of the human monoclonal antibody CP-751,871 (figitumumab) in patients with advanced ES, 2/2 responses and 6/8 disease stabilizations were observed[196]. Since IGF1R blockage in combination with chemotherapeutics showed good results in preclinical settings, clinical studies addressing the combination treatments have been initiated and are still ongoing.

However, huge expectations from IGF1R antagonists were not fulfilled so far, since only a minority of patients with advanced ES responds to IGF1R antagonists. Why is this the case is still unclear. There is a need for predictive molecular markers that would define the subset of patients likely to respond. Identification of such biomarkers would be beneficial for future clinical development of IGF1R antagonists since it would enable pre-selection of responsive patient populations[204]. Expression levels of IGF1R, IGF1, IGFBPs, are the main candidate biomarkers along with activation of downstream signaling effectors such as AKT[205-206]. Interestingly, genetic mutations within the IGF1R signaling pathway as molecular basis for sensitivity, have not been detected so far[182]. It is possible that alternative signaling inputs bypass targeted IGF1R inhibition downstream of the cell surface receptor since similar resistance mechanisms have already been

observed in rhabdomyosarcoma mouse model[205]. Among these, co-activation of RTKs such as epidermal growth factor receptor (EGFR) and ERBB2 has been observed. Combined targeted treatment of both RTKs elicited enhanced antitumor activitiy[206-207]. Therefore, combined targeting strategies are now under clinical investigation.

The future clinical development of IGF1R blocking agents for ES patients is under question. The main reason is low efficacy of IGF1R blocking agents observed in frequent cancer types such as breast cancer, colon carcinoma, and non-small cell lung cancer (NSCLC), causing decreased commercial interest in further development. Nevertheless, at the moment there are 82 IGF1R antagonist studies with 25 still recruiting (http://www.clinicaltrials.gov). Future development will depend on the outcome of these ongoing studies.

4.4.2.4 Targeting other receptor tyrosine kinases

Targeting several RTKs in other types of cancer showed very good results, such as the use of imatinibin the treatment of patients with chronic myelogenous leukemia (CML) and gastrointestinal stroma tumors (GISTs). Therefore, the expression and role of RTKs in ES was subject of several investigations[208-211]. In ES platelet-derived growth factor receptor-β (PDGFRB) is expressed and it is suggested that it plays a role in cell proliferation and metastasis. Inhibition of PDGFRB by RNAi and a specific kinase inhibitor, AG1295, has been shown to impair cell growth and chemotaxis in vitro and in vivo[197, 212]. However, since there is no detectable expression/secretion of PDGFRB ligands in ES, this suggests a dependence on paracrine stimulation from the microenvironment[212]. Also, in contrast to other malignancies, where activating PDGFRB mutations are common, no mutations have been described in ES so far[213]. Nevertheless, PDGFRB seems to be a good candidate for targeted therapy. However, there are no specific inhibitors exclusively inhibiting PDGFRB. The BCR-ABL inhibitor imatinib (Gleevec) has emerged as a multikinase inhibitor for ABL, KIT, and PDGFRs. Also second-generation multikinase inhibitors such as sunitinib (Sutent), sorafenib (Nexavar) nilotinib (Tasigna), and dasatinib (Sprycel) are effective against PDGFRB[207]. However, the relevance of targeting the PDGF/PDGFRB axis in ES remains unclear, since only multikinase inhibitors have been used so far. c-KIT, receptor for stem cell factor (SCF), is another target of imatinib. It is expressed in over 30% of ES[214]. Despite expression, there was no significant effect observed[213]. In vitro, imatinib alone did not induce significant apoptosis, but had synergistic effect with vincristine and doxorubicin[215]. One reason might be that in gastrointestinal stromal tumors, where Imatinib is

effective, mutations render Kit constitutively active. In ES these kind of activating mutations are rare. In two clinical trials Imatinib has been evaluated as single agent. In these phase II studies in sarcomas, 0/13 and 1/24 ES patients responded[216-217]. Therefore, it is hard to say whether PDGFRB and/or KIT present relevant targets in ES.

4.4.2.5 Targeting intracellular kinases

Activation of the mammalian target of rapamycin (mTOR) pathway is a very common event during tumorigenesis of several types of cancer[218]. In particular, strong preclinical evidence suggests a pivotal role of this pathway during the sarcomagenesis. Therefore, in childhood sarcoma models it was shown that the mTOR inhibitor rapamycin has activity against tumor growth[219], which is probably a result of at least partial down-regulation of vascular endothelial growth factor (VEGF) expression and as consequence inhibition of angiogenesis[195]. Interestingly, in ES rapamycin is not only blocking cell proliferation in vitro, but was also shown to decrease EWS-FLI1 fusion protein expression[220]. At the moment there are several rapamycin derivatives in clinical development in phase I/II studies in childhood sarcomas, including RAD001 (everolimus), CCI-779 (temsirolimus), and AP23573 (ridaforolimus). However, one problem with rapamycin is that inhibition of mTOR induces an IGF1R dependent feedback activation of AKT thus attenuating its effects[195, 197, 205, 218]. Possible combined inhibition of mTOR and IGF1R might be a logical step forward. Upon showing such synergistic effect of combined IGF1R inhibition and rapamycin in vitro as well as in vivo in different childhood sarcomas[195], clinical phase I/II studies have been initiated and first results suggest a favorable toxicity profile[221] (http://www.clinicaltrials.gov).

Taken together, it is clear that even though there is an increasing knowledge about biology of ES, there is still lack of transfer of this knowledge to the clinics. Therefore, it is necessary to elucidate all the potential contributors to the pathogenesis of ES that would enable establishment of successful targeted therapy.

4.5 Aims of the thesis

Given the fact that EWS/FLI1 is crucial for both ES development as well as tumor maintenance it was of great interest to establish an approach that would enable us to find compounds/inhibitors with activity against ES cells. Existing screening strategies did not show to be optimal for the discovery of inhibitors of EWS/FLI1 activity/expression. Therefore, the first aim of this thesis was to establish an expression based read-out system to measure modulation of transcriptional activity of EWS/FLI1 for small molecule discovery. The idea was to monitor activity of EWS/FLI1 by measuring expression of several EWS/FLI1 target genes and at the same time to measure proliferation of cells. Second aim was to apply this screening approach on a library of mainly FDA approved compounds to find compound(s) that can induce apoptosis in ES cells, followed by validation experiments including testing the effect of compound in xenografted mice. The third aim was to use the established screening approach to screen a small library that includes a broad range of kinase inhibitors thus covering all major signalling pathways in order to identify the molecular pathway(s) that may contribute to the transcriptional activity and/or expression of EWS/FLI1.

5 Results

5.1 Manuscript 1: Small-molecule screen identifies modulators of EWS/FLI1 target gene expression and cell survival in Ewing's sarcoma

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Manuscript published in International Journal of Cancer on February 9, 2012

Contribution: I performed all experiments except meta analysis of gene expression data (Figure 1A), drug screening was done together with FR (Figure 3), in vitro assays (Figure 4A and B) and midostaurin treatment assays (Figure 5) were done by KP. I was further responsible for statistical analysis, data interpretation as well as writing and formatting of this manuscript.

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Small-molecule screen identifies modulators of EWS/FLI1 target gene expression and cell survival in Ewing's sarcoma

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Ewing's sarcoma family of tumors (EFT) is characterized by the presence of chromosomal translocations leading to the expression of oncogenic transcription factors such as, in the majority of cases, EWS/FLI1. Because of its key role in Ewing's sarcoma development and maintenance, EWS/FLI1 represents an attractive therapeutic target. Here, we characterize PHLDA1 as a novel direct target gene whose expression is repressed by EWS/FLI1. Using this gene and additional specific well-characterized target genes such as NROB1, NKX2.2 and CAV1, all activated by EWS/FLI1, as a read-out system, we screened a small-molecule compound library enriched for FDA-approved drugs that modulated the expression of EWS/FLI1 target genes. Among a hit-list of nine well-known drugs such as camptothecin, fenretinide, etoposide and doxorubicin, we also identified the kinase inhibitor midostaurin (PKC412). Subsequent experiments demonstrated that midostaurin is able to induce apoptosis in a panel of six Ewing's sarcoma cell lines *in vitro* and can significantly suppress xenograft tumor growth *in vivo*. These results suggest that midostaurin might be a novel drug that is active against Ewing's cells, which might act by modulating the expression of EWS/FLI1 target genes.

The Ewing's sarcoma family of tumors (EFT) is a group of malignancies affecting bones and soft tissue in adolescents. Its hallmark is the unique presence of an EWS/ETS gene rearrangement leading to the expression of chimeric proteins known to play a role in transcriptional regulation[1] and in RNA processing.[2] The most frequent fusion product associated with about 85% of EFT, EWS-FLI1, is the founding member of a whole class of similarly structured chimeric proteins associated with a variety of human sarcomas, specific types of leukemias and prostate carcinomas.[3,4] The expression of EWS/FLI1 protein constitutes a rate limiting step in Ewing's sarcoma oncogenesis. Hence, EWS/FLI1 is highly associated with EFT, and its transforming and tumorigenic potential was experimentally confirmed.[5–9] Besides its importance in the onset of tumorigenesis, continued expression of EWS/FLI1 is necessary for tumor maintenance.[9]

The product of the fusion gene is thought to act as a transcription factor that combines EWS and FLI1 properties to generate a chimeric protein with new transactivation potential.[10,11] Furthermore, EWS/FLI1 may deregulate target genes at the posttranscriptional level most likely by interacting with RNA polymerase II complex[12–14] as well as with proteins known to play a role in splicing[15,16] or in RNA degradation.[17,18] An oncogenic contribution by the wild-type ETS counterparts is excluded as it was shown that overexpression of wild-type ETS proteins competes with the transforming activity of EWS/ETS.[8]

Oncogenic fusion proteins are very attractive drug targets, because modulation of their activity should affect specifically oncogenic cells while preserving healthy cells. However, drugs directly targeting transcription factors are not available until today. Compounds available or underdevelopment against Ewing's sarcoma act mostly on growth factor pathways (mTOR, IGF1R pathway) or histone deacetylation.[19–23] As an interesting alternative, it might be possible to target the activity of EWS/FLI1 either directly or indirectly. One example for such a strategy was provided recently[24] with the identification of ARA-C, which was suggested to reduce EWS/FLI1 levels. Here, we extended this strategy and used a combination of well-defined and characterized EWS/FLI1 target genes

Key words: Ewing's sarcoma, EWS/FLI1, drug screen, midostaurin, PHLDA1

Abbreviations: CCND1: cyclin D1; ChIP: chromatin immunoprecipitation; DMSO: dimethylsulfoxide; EFT: Ewing's family of tumors; ETS: E-twenty six; mTOR: mammalian target of rapamycin; PHLDA1: pleckstrin homology-like domain family A member 1; RT-PCR: reverse transcriptase polymerase chain reaction; SPRY2: sprouty homologue 2; TUNEL: deoxynucleotidyl transferase doxyuridine triphosphate nick end labeling

Additional Supporting Information may be found in the online version of this article.

*A.B. and K.P. contributed equally to this work

Grant sponsor: Swiss National Science Foundation; **Grant number:** 31003A-118035; **Grant sponsor:** Swiss Research Foundation Child and Cancer

DOI: 10.1002/ijc.27472

History: Received 14 Jul 2011; Accepted 17 Jan 2012; Online 9 Feb 2012

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to screen a small molecule compound library of mostly FDA-approved drugs. Apart from drugs with a well-known activity profile in Ewing's sarcoma, we also found a kinase inhibitor (midostaurin) with broad spectrum of targets as a promising drug for this pediatric tumor. These data open new perspectives for the treatment of Ewing's sarcoma using small-molecule kinase inhibitors.

Material and Methods

Cell lines, pharmacological inhibitors and plasmids

Four type 1 (A673, SKNMC, TC71, SKPNDW) and two type 2 Ewing's cell lines (SKES, RDES) were used. The SKPNDW cells were kindly provided by Prof. Javier Alonso (IIB, Madrid), TC71 cells by Prof H. Kovar (St-Anna Children's Hospital, Vienna, Austria) and SKES and RDES by Prof. K.L. Schaefer (Institute of Pathology, Duesseldorf, Germany). A673 cells were purchased from the American Type Culture Collection. Cells were cultivated on 0.1% gelatine-coated plates (Sigma-Aldrich, Buchs, Switzerland) in RPMI medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine. All cells were cultivated at 37°C in 5% CO_2. Midostaurin was provided by Novartis (Basel, Switzerland). For ectopic expression and construction of FLAG-tagged EWS/FLI1, the most prevalent type 1 EWS/FLI1 fusion cDNA was amplified[25] and inserted inserted into p3XFLAG-CMV-14 (Sigma-Aldrich) using Not1 and EcoRV restriction enzymes.

LOPAC^{1280TM} screening assay

About 10^4 cells were plated in 96-well plates 24 hr before treatment. LOPAC^{1280TM} compounds were added to cells in complete RPMI medium at a final concentration of 5 μM for 24 hr. Lysis and subsequent cDNA synthesis were performed using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies) according to the manufacturer's protocol. Finally, quantitative reverse transcription PCR (qRT-PCR) with corresponding target probes was performed as described below. Similarly treated 96-well plates were used to measure cell viability in parallel using WST-1-cell proliferation kit (Roche Applied Sciences, Rotkreuz, Switzerland).

Drug treatment, proliferation and apoptosis assay

Drug treatments were performed in 96-well plates after seeding 5×10^3 cells per well 24 hr before. Cells were treated with the pharmacological inhibitors in a volume of 100 μl serum-containing medium. Viability of cells was assessed using the cell proliferation kit (WST-1; Roche Applied Sciences, Rotkreuz, Switzerland). Annexin-V stainings were performed with FITC-labeled annexin-V and propidium iodide (PI; BD Biosciences, San Jose, CA). Briefly, cells were trypsinized and washed in cold PBS, and 5×10^5 cells were resuspended in 100 μl binding buffer (10 mM HEPES/NaOH, pH 7.4 and 140 mM $NaCl_2$). Annexin-V-FITC and PI (5 μl each) were added and incubated at 4°C in the dark for 30 min before flow cytometry analysis (Beckman Coulter Cytomics FC500; Hialeah, FL). Caspase activity was detected by CaspGLOW Red Active caspase-3 Staining Kit (Alexis, Lausen, Switzerland) according to the manufacturer's instructions. Briefly, 1 μl specific substrate for caspase-3 (Red-DEVD-FMK) was added to 3×10^5-treated cells in 300 μl of medium for 1 hr at 37°C, and enzymatic cleavage was measured using the multidetection microplate reader Synergy HT (Bio-Tek Instrument, Winooski, USA).

Cell-cycle analysis

Cells were detached by trypsinization (0.05% Trypsin, w/ EDTA, Invitrogen Life Technology, Basel, Switzerland), washed in PBS and 1×10^5 cells were fixed and stored in 70% ethanol at −20°C. For cell-cycle measurement, cells were washed in PBS, resuspended in 300 μl PI (50 μg/ml PI and 3.5 μg/ml RNase in PBS) and incubated at 4°C in the dark for 30 min before flow cytometry analysis (Beckman Coulter Cytomics FC500; Hialeah, FL). Data were processed with FlowJo software (Treestar, Ashland, OR).

Silencing of EWS/FLI1

A total of 1.5×10^5 A673 cells were seeded in a six-well plate. After 24 hr, transfection was carried out using N-TER^{TM} nanoparticle reagent (Sigma-Aldrich) and 40 nM (final concentration) small interfering RNA (siRNA) of FLI1 (Hs_Fli1_1 HP siRNA, Qiagen, Hombrechtikon, Switzerland, 5′-CAGGACATATGTGGCCTTGAA-3′) or EWS/FLI1 (5′-GGCAGCAGAACCCUUCUUA-dCdG-3′).[26] As controls, glyceraldehyde-3-phosphate dehydrogenase (Silencer FAM labelled GAPDH, Ambion, Austin, TX) and negative control #1 siRNA (Ambion) were used (sequences proprietary of manufacturer). Cells were lysed 24 hr after silencing if not mentioned otherwise in lysis buffer containing 50 mM NaH_2PO_4 (pH = 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM EGTA supplemented with protease inhibitor cocktail (Complete + 1 mM EDTA, Roche).

Luciferase assays

Tpro1 + 2 and Tpro2 plasmids were kindly provided by Thomas Hinz, and they are described in detail previously (Meier-Noorden, Flindt, 2004). Promoter region of Phlda1 was subcloned from these plasmids using HindIII enzyme into pGL4.19 luciferase vector (Promega). Using site-directed mutagenesis kit (Invitrogen), promoter sequence within Tpro1 + 2 plasmid was mutated at positions −1774/−1771, −1347/−1344, −1165/−1162, −547/−544 and −347/−344 (position relative to the transcription initiation site), where these sequences were changed into AAAA and thus generated five mutant reporter vectors, namely mutETS1, mutETS2, mutETS3, mutETS4 and mutETS5. About 3.5×10^5 of HEK293 cells or A673 cells were plated in six-well plates and 24 hr later transfected using Lipofectamin 2000 (Invitrogen) with 1 μg of reporter construct, 800 ng of EWS/FLI1 expression plasmid (kindly provided by H. Kovar) or pcDNA3.1. The cells were also transfected with 40 ng of pSV-β gal for

standardization of transfection efficiency. After 24 hr, cells were lysed and assayed for luciferase activity using the luciferase reporter assay system (Promega) according to manufacturer's instructions. Measured activity of β-galactosidase was used for the normalization of luciferase activity.

ChIP

ChIP was performed on 10^7 A673 cells fixed with 1% formaldehyde for 10 min using ChIP IT Express Kit (Active Motif) according to manufacturer's protocol with some modifications. Briefly, additional washing buffer was used containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, 500 mM NaCl at pH8.1. Cross-links were reversed for 15 hr at 65°C. The chromatin was purified and analyzed by PCR (32 cycles) using specific primers (for. 5′-TGTGAGCGTCGG CAGTAGTC-3′, Rev-5′-CGCACGCCTCATTAACTTGG-3′).

Real-time PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed under universal cycling variables on an ABI 7900 instrument using commercially available target probes and mastermix (all from Applied Biosystems, Rotkreuz, Switzerland). Detection of EWS/FLI1 was achieved using EWS forward (5′-GTCAACCTCAATCTAGCACAGGG-3′, 330 nM) and FLI1 reverse (5′-CTGTCGGAGAGCAGCTCCAG-3′, 330 nM) primers and probe (5′-CTCCTA CCAGCTATTCCTCT ACACAGCCGACT-3′, 100 nM) under conditions: 2 min 50°C/10 min 95°C/50× (10 sec 95°C/1 min 66°C). Data were analyzed using SDS 2.2 software. CT values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression levels of the target genes were calculated using the $\Delta\Delta C_T$ method. All experiments were performed in triplicate and repeated at least three times. Data analysis was done with the GraphPad prism software (San Diego, CA) and statistical analysis using the Student's *t*-test. Commercially available target probes used are listed below (Applied Biosystem): PHLDA1:Hs00378285_g1, CAV1:Hs00184697_m1, IL1RAP: Hs00370506_m1, ARHGAP1:Hs00188815_m1, POU4F:Hs00366 711_m1, NKX2.2:Hs00159616_m1, SPRY2:Hs00183386_m1, CCND1:Hs00277039_m1, PTGER3:Hs00168755_m1, NR0B1: Hs03043658_m1, GAPDH:Hs99999905_m1.

Microarray expression analysis

Normalization and comparison of open source CEL files were done using algorithms from RACE (www.race.unil.ch). Affymetrix probeset level of cell lines was RMA normalized, and tumor affymetrix data were MAS 5.0 normalized. In the context of Bolstad *et al.*,[27] MAS5.0 and RMA are equivalent in finding significant genes at higher expression levels. Fold change for cell-line analysis was set to 2.0 with *p*-value of 0.05. Fold change for tumor sample analysis was set to 3.5 and *p*-value 0.001.

In vivo antitumor activity of midostaurin

When cells reached 70% confluence, they were trypsinized into single-cell suspensions. After washing once with PBS, Ewing's sarcoma cells (0.5×10^6 A673; 3×10^6 SKES; 3×10^6 TC71) diluted in 100 µl of PBS were subcutaneously injected into the left flank of 4–8-week-old NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (The Jackson Laboratory, Bar Harbor, USA) mice (male and female). In all mice, palpable tumors were observed 2–3 weeks after inoculation. Once tumor volume reached 50–150 mm^3, mice were randomly assigned to receive oral gavage with midostaurin (100 mg/kg) or vehicle alone (five or eight mice per group). Doses of midostaurin were administered by gavage daily for 10 days in the case of A673 xenografts. Because of a change in the formulation of the drug for *in vivo* use, minor alterations of the protocol were implemented. Hence, in the case of SKES and TC71 xenograft mice, midostaurin was administered every second day for 2 weeks. The dosage of the drug was obtained from previously reported studies[28] and was also recommended by the manufacturer. Tumor dimensions were measured every 1 or 2 days with digital calipers to obtain two diameters of the tumor. The tumor volume was determined by the formula $(D \times d^2/6) \times \pi$, where D is the longer diameter and d is the shorter diameter. After treatment, mice were sacrificed and tumors extracted for further analysis. Autopsy was performed on each mouse to exclude the presence of major general toxicity events. Xenograft studies were approved by the Cantonal Veterinary Office of Zurich, and all animal care was in accordance with the existing Swiss legislation and guidelines.

Immunohistochemistry

Tumors obtained by dissection from sacrificed mice were fixed, and immunohistochemical analysis was done as described before (Amstutz, 2008). H&E, Ki67, CD31 and cleaved Caspase 3 were stained. For quantitative evaluation, the number of positive cells was counted in five randomly selected visual fields in non-necrotic areas of the tumor using Image J software. Two-tailed, unpaired *t* test was used for statistical analysis. The level of significance was set at $p < 0.05$.

Immunoblotting

Cells were washed twice with PBS and harvested in lysis buffer-containing 50 mM NaH_2PO_4 (pH = 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM EGTA supplemented with protease inhibitor cocktail (complete + 1 mM EDTA, Roche). Protein concentration was determined by the method of Bradford (Biorad, Reinach, Switzerland). About 10–30 µg of protein extract was resolved on 10% SDS–PAGE and transferred onto nitrocellulose membrane (Whatman, Germany). Primary antibodies were used as follows: anti-FLI1 rabbit polyclonal antibody C-19 (Santa Cruz Biotechnologies, Santa Cruz, USA) (dilution 1:300), anti-PARP rabbit polyclonal antibody (Cell Signalling Technology, Beverley, MA; dilution 1:1,000), FLAG antibody (M2, Sigma; dilution, 1:1,000) and anti-β-tubulin I mouse monoclonal antibody (Sigma-Aldrich; dilution 1:40,000). After incubation with the appropriate secondary peroxidase-conjugated antibodies (1:1,000), detection was performed with the ECL chemiluminescent

reagent (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions.

Immunofluorescence

A total of 0.5×10^6 A673 cells per well were seeded in a 35-mm plate. After 24 hr, transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with FLAG tagged EWS/FLI1 and 24 hr after transfection a total of 3×10^4 transfected cells were seeded on gelatine-coated cover slides. After 24 hr, cells were treated O/N with 500 nM of midostaurin followed by immunostaining. Cells were fixed for 20 min in 4% paraformaldehyde, washed with PBS and stained using mouse monoclonal primary antibody against FLAG (dilution 1:200) and the appropriate secondary antibody coupled to Alexa 488 (dilution 1:300). Nuclei were stained with ProLong® Gold antifade reagent-containing DAPI (Invitrogen).

Results

Meta-analysis identifies PHLDA1 as a new target gene repressed by EWS/FLI1

A number of target genes have been described recently to be positively modulated by EWS/FLI1, but only few repressed genes have been characterized in detail. As an output to assess transcriptional activity of EWS/FLI1, an ideal monitoring system should include target genes that are both induced as well as repressed. To identify new target genes repressed by EWS/FLI1, we performed a meta-analysis of publicly available microarray datasets. Three lists of significantly differentially expressed probesets were generated by comparing publicly available data from silenced *versus* nonsilenced EWS/FLI1 in A673, EW24 and SKNMC cells,[19,26] which were generated by different silencing methods (siRNA and shRNA). Thirty-two genes were found to be commonly regulated in all three cell lines (Fig. 1*a*, left). As cell lines grown in culture may have acquired additional mutations and epigenetically induced changes in gene expression, we further compared the list of 32 genes to data derived from patient samples of Ewing's sarcoma, neuroblastoma, rhabdomyosarcoma and acute lymphoblastic leukemia (ALL[29–31]; see Material and Methods). Three hundred and thirteen probesets were identified to be differentially expressed in Ewing's patients with a p-value of <0.001 and a fold change of >3.5 compared to the other tumor types (Fig. 1*a*, right). Finally, the gene lists from the cell-culture systems and biopsies were combined, and six genes could be identified with the three top ranking genes being IL1RAP, PHLDA1 and ARHGAP. To validate our results, we silenced expression of EWS/FLI1 in A673 cells using siRNA targeting the fusion protein breakpoint. This resulted in downregulation of EWS/FLI1 mRNA by 81 and protein by 84% (Fig. 1*b*). In silenced A673 cells, PHLDA1 expression was induced by fourfold ($p < 0.0001$), and IL1RAP mRNA expression was repressed by more than twofold ($p < 0.003$; Fig. 1*c*), indicating high responsiveness to EWS/FLI1 protein downregulation. Additionally, silencing of EWS/FLI1 was able to modulate the mRNA levels of other selected, already known target genes of EWS/FLI1 such as NKX2.2, CAV1, CCND1, POU4F and SPRY2 (Fig. 1*d*). Hence, our meta-analysis identified PHLDA1 as a novel gene repressed by EWS/FLI1.

PHLDA1 is repressed by direct binding of EWS/FLI1

To assess whether EWS/FLI1 repressed PHLDA1 expression directly, we cloned 2.2 kb of the PHLDA1 promoter upstream of a luciferase reporter gene. Cotransfection of this reporter construct along with increasing amounts of a EWS/FL1 expression plasmid in HEK293 cells demonstrated a dose-dependent repression of luciferase activity by greater than threefold (Fig. 2*a*). Similarly, cotransfection of the luciferase reporter with EWS/FLI1 resulted in repression of luciferase activity compared to empty vector control (Fig. 2*b*).

Closer inspection of the PHLDA1 promoter revealed five potential ETS-binding sites distributed in the 2.2-kb promoter region (Fig. 2*c*). To identify the sites important for PHLDA1 repression, several deletion constructs of this region were generated (Fig. 2*d*). Measurement of luciferase activity revealed that binding sites in the entire 2.2 kb region contribute to repression (Fig. 2*e*). To determine exactly which ETS sites might be involved in EWS/FLI1-mediated repression of the PHLDA1 promoter, we mutated all five ETS sites to AAAA (Figs. 2*f* and 2*g*). Mutation of ETS3, ETS4 and ETS5 abolished repression of the reporter activity, while this was not the case for mutations in ETS1 or ETS2. The highest induction of expression (threefold) was observed after mutation of ETS4.

To further substantiate this notion, we used chromatin immunoprecipitation to analyze binding of EWS/FLI1 to the PHLDA1 promoter *in vivo* in A673 cells. Using a fragment containing the ETS4 site of the PHLDA1 promoter (Fig. 2*h*), with an anti-EWS/FLI1, antibody resulted in an immunoprecipitated fragment not seen with the anti-His or anti-IgG control antibodies (Fig. 2*i*). We also did not observe specific immunoprecipitation with a negative control promoter (Fig. 2*i*). Altogether, these results indicate that EWS/FLI1 is able to specifically bind the PHLDA1 promoter both *in vitro* and *in vivo*, thereby allowing direct repression of its expression.

Screening of a chemical library identifies midostaurin as a modulator of EWS/FLI1 target gene expression

Using PHLDA1 expression together with the well-described target genes CAV1 and NROB1 as a read-out system, we screened a library of 1,280 drug-like and well-annotated compounds (LOPAC^{1280TM}, Sigma) covering all major drug types to identify drugs that would be able to modulate EWS/FLI1 target gene expression. We also included doxorubicin, standard regimen used in therapy of Ewing's sarcoma together with midostaurin, a kinase inhibitor shown previously to have activity against sarcomas.[28] Such a system encompassing

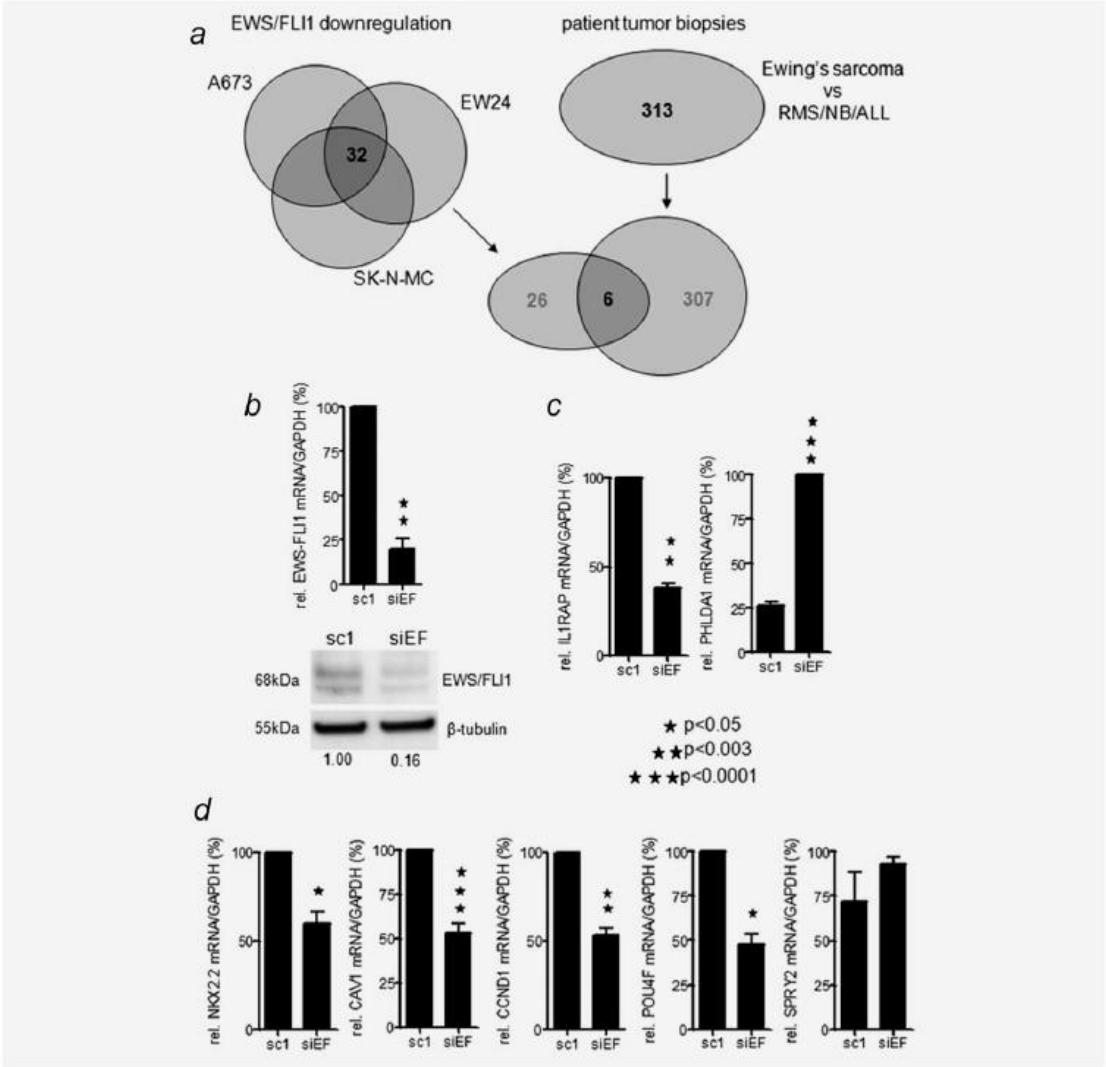


Figure 1. Meta-analysis identifies PHLDA1 as a gene repressed by EWS/FLI1. (*a*) Analysis of published gene expression data of EWS24, SKNMC and A673 cells silenced for EWS/FLI1 compared to their respective scrambled controls (left panel). Tumor biopsy samples from patients with Ewing's sarcoma were compared to other small blue round cell tumors and leukemia biopsies (right panel). The number of common genes shared between the different analyses appears in bold. Combination of both analyses (right and left upper panels) is indicated in the lower panel. (*b*) Analysis of EWS/FLI1 expression levels in A673 cells after silencing compared to control was performed by qRT-PCR from total RNA (upper panel) or by immunoblot from protein extracts (lower panel, quantification by densitometry in % is indicated below (*c*, *d*) EWS/FLI1 target gene expression levels in A673 cells after treatment with EWS/FLI1 specific or scrambled siRNA was assessed. Statistical analysis using paired *t*-test. **p*-value < 0.05, ***p*-value < 0.003. $N = 4$ for EWS/FLI1, NKX2.2, CCND1, POU4F1, SPRY2, PHLDA1 and $N = 5$ for CAV1, $N = 3$ for IL1RAP and qRT-PCR data are normalized to GAPDH mRNA expression.

both overexpressed and repressed target genes is expected to stably monitor fusion protein activity. Upon treatment of cells at a final drug concentration of 5 μM for 24hr (Fig. 3*a*) and based on 20% downregulation of either the fusion protein and/or NR0B1 and a 50% upregulation of PHLDA1 measured using in plate quantitative RT-PCR, we identified 32 compounds as first hits. To validate these hits, we proceeded to a second screening (Supporting Information Table 1 and Fig. 1), which was done in duplicate and included three additional cell lines (RDES, SKNMC and TC71)

together with two additional target genes (NKX2.2 and CAV1) and parallel cytotoxicity measurements. The final hit-list of compounds was based on a significant ($p < 0.05$, unpaired two-tailed t-test) modulation of at least three of four target genes compared to the untreated controls in both A673 and RDES cells and is composed of 10 compounds (Fig. 3*b*) fulfilling these criteria in two cell lines. Among these top hits, the majority of compounds are well known and widely used chemotherapeutic agents such as camptothecin, idarubicine and etoposide, which are already used in first line treatment of Ewing sarcoma either alone or in combination.[32,33] This finding directly validates our screening strategy in an unbiased way. Interestingly, also the broad spectrum kinase inhibitor midostaurin showed significant modulation of all four EWS/FLI1 target genes in A673 cells and of three target genes in RDES cells. Furthermore, all compounds including

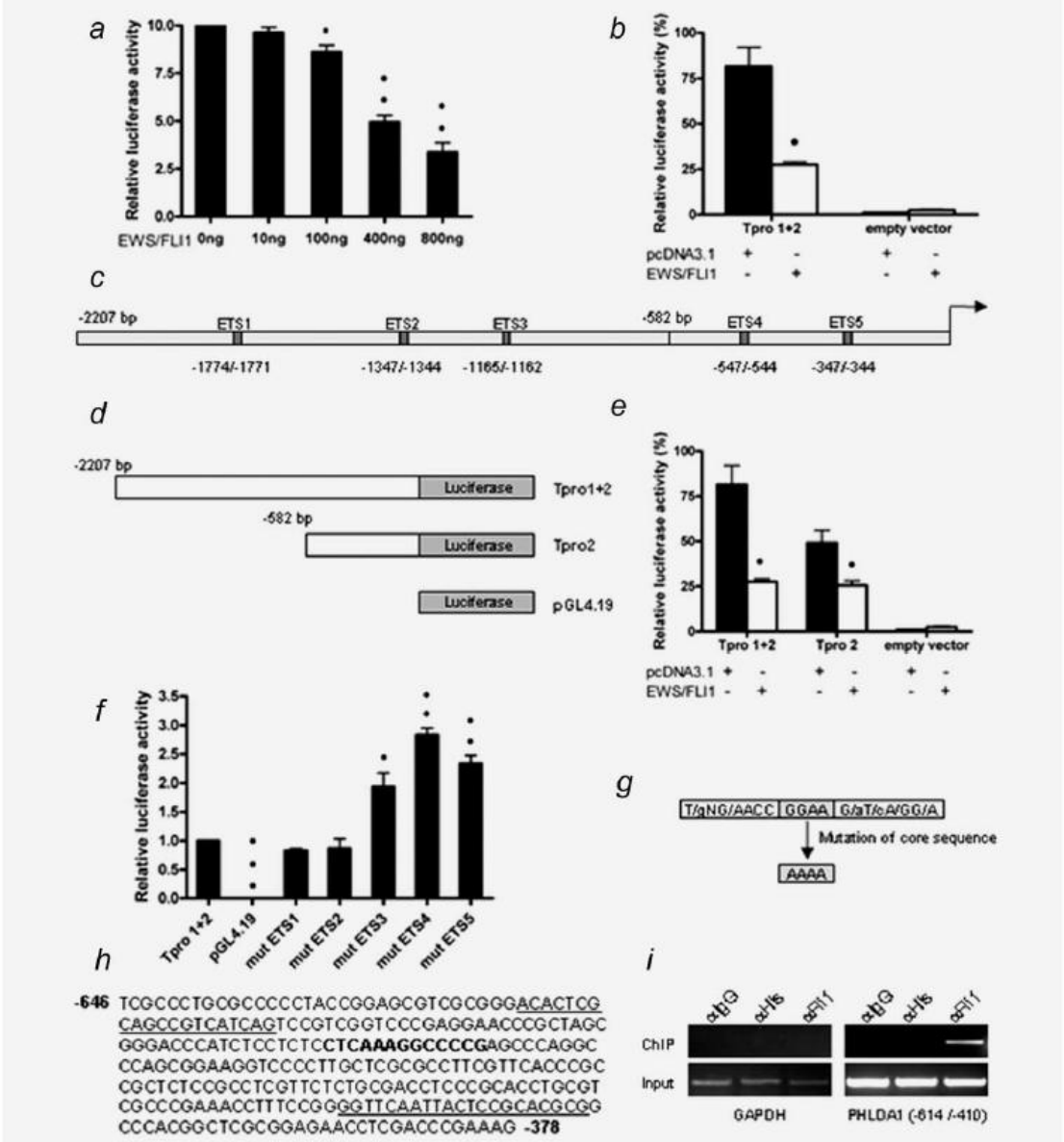
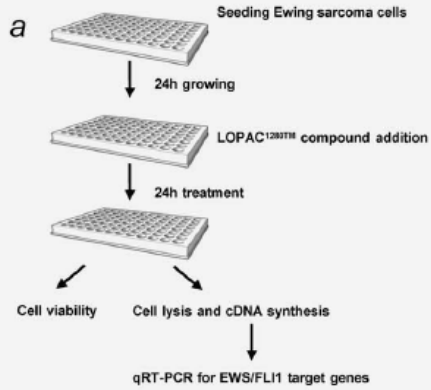


Figure 2.

a



b

	Gene modulation in A673 and RDES Ewing's sarcoma cells*											
	EWS/FLI1		NR0B1		PHLDA1		NKX2.2		CAV1		Proliferation*	
Compound[a]	A673	RDES	A673	RDES	A673	RDES	A673	RDES	A673	RDES	A673	RDES
Camptothecin	**67**	**38**	**72**	**1**	**446**	**988**	**31**	**20**	**24**	**7**	81	63
PKC412	79	**70**	**80**	70	**193**	**299**	60	88	**67**	**74**	87	56
Retinoic acid p-hydroxyanilide	**83**	82	**74**	89	**469**	**540**	56	**22**	70	**33**	93	62
Etoposide	95	159	131	46	**320**	**902**	67	**66**	**78**	**78**	96	74
Idarubicin	**29**	17	**9**	1	14	274	16	10	74	39	92	74
N-Oleoyldopamine	64	31	**64**	197	**423**	536	17	25	50	21	90	81
5-Bromo-2'-deoxyuridine	82	150	**51**	23	152	194	54	111	79	72	99	121
K114	74	117	**28**	244	**196**	396	31	16	75	56	92	82
Doxorubicin	**11**	**8**	**2**	**1**	2	18	**5**	**7**	**47**	**41**	40	8
Fluphenazine dihydrochloride	169	375	**35**	1	**529**	1	1	54	96	62	90	74

[a] Treatment: 5 μM of compound for 24 h
* in % of control (=100%)
** p<0.05, unpaired two-tailed t-test, significant values written in bold

Figure 3. Drug screen for the identification of modulators of EWS/FLI1 transcriptional activity. (*a*) Schematic methodology of the screening procedure. (*b*) Modulation of relative expression levels of EWS/FLI1 and specified target genes NR0B1, PHLDA1, NKX2.2 and CAV1. A673 and RDES cells were treated with compounds from the LOPAC1280TM library at a final concentration of 5 μM for 24 hr. Cell proliferation measurement using WST-1 assay was performed in parallel. Values are shown in percentage of solvent control (PBS/DMSO = 100%) and represent the mean of two to four independent experiments performed in duplicate. Significant values are written in bold ($p < 0.05$, unpaired two-tailed Student's *t*-test).

Figure 2. EWS/FLI1 is downregulating PHLDA1 by direct binding to its promoter region. (*a*) 293 HEK cells were cotransfected with reporter plasmid containing a 2.2-kb fragment of the PHLDA1 promoter (Tpro1 + 2), a pSVβ-galactosidase expression plasmid used as a control for transfection efficiency and with varying amount of EWS/FLI1 expression vector. Cells were collected after 24 hr and assayed for luciferase activity. On the *y* axis, light intensity in relative light units (RLU) is shown (mean ± standard error for three independent experiments). (*b*) Relative luciferase activity for 293 cells cotransfected with luciferase reporter (Tpro1 + 2) and either EWS/FLI1 expression plasmid or empty expression vector (pcDNA3.1). As a control, empty luciferase vector (pGL4.19) is used. (*c*) Schematic representation of PHLDA1 promoter with designated potential ETS sites. Numbers represent position relative to the transcription initiation site. (*d*) Schematic representation of PHLDA1 promoter reporter constructs used in luciferase assays. (*e*) Transfection of A673 cells with PHLDA deletion constructs and EWS/FLI1 or empty vector. Relative luciferase activity in % is shown after normalization to β-galactosidase. (*f*) A673 cells were cotransfected with the mutated constructs indicated and pSV-β-galactosidase as control for transfection efficiency. After 24-hr, luciferase activity was measured and expressed relative to the nonmutated control plasmid (Tpro1 + 2), which was set to 1. (*g*) Scheme of the consensus sequence of ETS site and site-directed mutagenesis of its core sequence that has been done for each of five potential ETS-binding sites. (*h*) Sequence of the PHLDA1 promoter that includes potential EWS/FLI1-binding site ETS4 (bold) showing also the position of primers (underlined) used in chromatin immunoprecipitation (ChIP) experiment. (*i*) ChIP experiments have been performed on A673 cell extracts using anti-FLI1 monoclonal antibody for immunoprecipitation of endogenous EWS/FLI1 and two different control antibodies (normal rabbit anti-IgG and antiHis antibody). PCR has been done and the 204 bp product analyzed on 3% agarose gel. As control, PCR on extracts before immunoprecipitation (input) is shown as well as amplification of GAPDH. Statistical analysis for all panels was done using unpaired *t*-test, *p-value < 0.01, **p-value < 0.001.

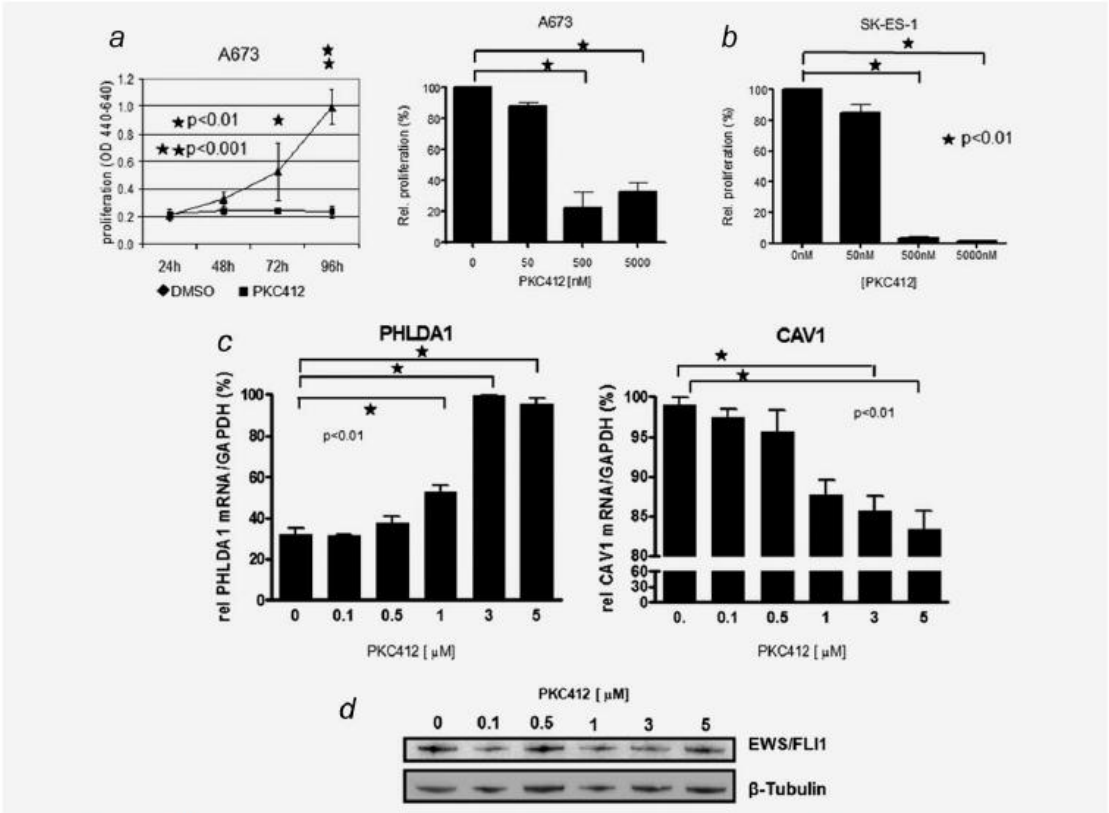


Figure 4. CAV1 and PHLDA1 expression is modulated by treatment with kinase inhibitor midostaurin. (*a*) Proliferation of A673 cells after 1, 2, 3 and 4 days of treatment with 500 nM midostaurin was determined by WST-1 assay, *Y* axis represents absolute proliferation units, results are shown as a mean ± SD ($N = 3$). (*b*) Viability of A673 cells treated with increasing concentrations of midostaurin for 48 hr, *Y* axis represents the relative proliferation in % compared to DMSO treatment ($N = 3$). A673 cells were treated with indicated concentrations of midostaurin for 24 hr. (*c*) mRNA expression of CAV1 ($N = 4$) and PHLDA1 ($N = 3$) as shown by qRT-PCR, data are normalized to GAPDH mRNA expression, **p*-value < 0.01. (*d*) Endogenous EWS/FLI1 protein expression in A673 cells upon midostaurin treatment for 24 hr on a western blot using a FLI1 antibody.

midostaurin showed a significant modulation of at least two or more target genes in two additional Ewing's sarcoma cell lines, SKNMC and TC71 (data not shown). Hence, these experiments identified known chemotherapeutic drugs together with fenretinide and the kinase inhibitor midostaurin as candidate modulators of EWS/FLI1 target genes. Midostaurin was subsequently characterized in more detail, because it has never been applied to Ewing's sarcoma before.

Midostaurin modulates expression of EWS/FLI1 target genes

To validate the activity of midostaurin on proliferation of Ewing's cell lines *in vitro*, we treated A673 and SKES1 cells with midostaurin in serum-containing medium. Complete inhibition of cell proliferation compared to DMSO control was observed after treatment with 500 nM kinase inhibitor over 4 days and dose-dependent inhibition at 48 hr (Figs. 4*a* and 4*b*). To confirm any potential influence of midostaurin treatment on the expression of EWS/FLI1 target genes, A673 cells were treated with increasing amounts of midostaurin for 48 hr, and expression of PHLDA1 and CAV1 mRNA was determined by quantitative RT-PCR. As expected, CAV1 mRNA expression decreased in the presence of midostaurin, whereas PHLDA1 expression increased in a dose-dependent manner (Fig. 4*c*), thereby confirming modulation of EWS/FLI1 target genes. Modulation might be due to decreased EWS/FLI1 protein levels *per se*. However, western blot analysis of the fusion protein revealed no change in protein concentration upon midostaurin treatment up to 5 µM (Fig. 4*d*). In addition, also nuclear localization of ectopically expressed

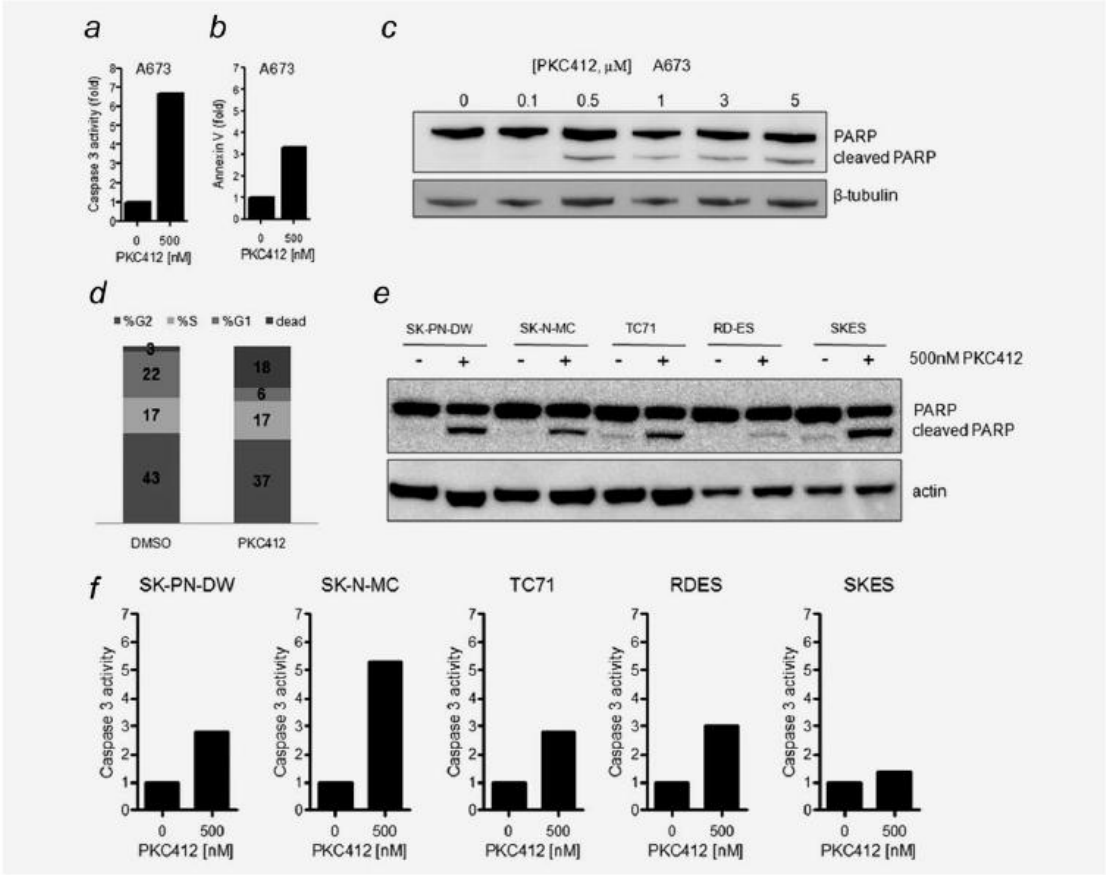


Figure 5. Midostaurin induces apoptosis in six Ewing's cell lines *in vitro*. Six different cell lines expressing either EWS/FLI1 type 1 (A673, TC71, SKNMC, SKPNDW) or type 2 (SKES1, RDES) were treated with kinase inhibitor midostaurin or DMSO control. (*a–e*) Induction of apoptosis by midostaurin in different Ewing's sarcoma cell lines was evaluated by measuring (*a*, *f*) caspase 3 activation (48 hr) ($N = 1$), (*b*) annexin V staining (24 hr), (*c*, *e*) PARP cleavage (24 hr) and (*d*) cell-cycle distribution (48 hr) ($N = 4$).

EWS/FLI1 in A673 cells was unchanged upon treatment (data not shown). Therefore, our results suggest that modulation of target gene expression involves other, yet uncharacterized mechanisms.

Midostaurin induces apoptosis in Ewing's cell lines *in vitro*

Ewing's cells are known to undergo apoptosis upon silencing of EWS/FLI1 by oligonucleotides or shRNA. This prompted us to investigate the influence of midostaurin on apoptosis. After 24 hr of treatment with 500 nM midostaurin, A673 cells displayed first signs of characteristic apoptotic morphology (data not shown). Caspase 3 activity and Annexin V staining increased three to sixfold following treatment, suggestive of apoptotic cell death (Figs. 5*a* and 5*b*). Dose-dependent activation of apoptosis was confirmed further by PARP cleavage (Fig. 5*c*). Moreover, cell-cycle analysis showed an increase in the sub-G1 and a decrease in G1 population, whereas percentage of cells in *S* and *G2* phase remained unchanged (Fig. 5*d*) suggesting induction of apoptosis. Similar results were obtained in five additional Ewing's cell lines (SKES, RDES, TC71, SKNMC and SKPNDW) carrying either EWS/FLI1 type 1 or type 2 as shown by enhanced caspase 3 activation and PARP cleavage (Figs. 5*e* and 5*f*). These results demonstrate that the kinase inhibitor midostaurin is able to efficiently induce apoptosis in a range of Ewing's sarcoma cells *in vitro*.

Tumor growth is inhibited by midostaurin treatment *in vivo*

As kinase inhibitors are interesting molecules for the potential treatment of Ewing's sarcoma, we further investigated the effects of midostaurin on tumor growth *in vivo*. Toward this end, different Ewing's cell lines (A673, SKES and TC71) were grown s.c. as xenografts in mice and treated with midostaurin (100 mg/kg) by oral administration for up to 14 days. In

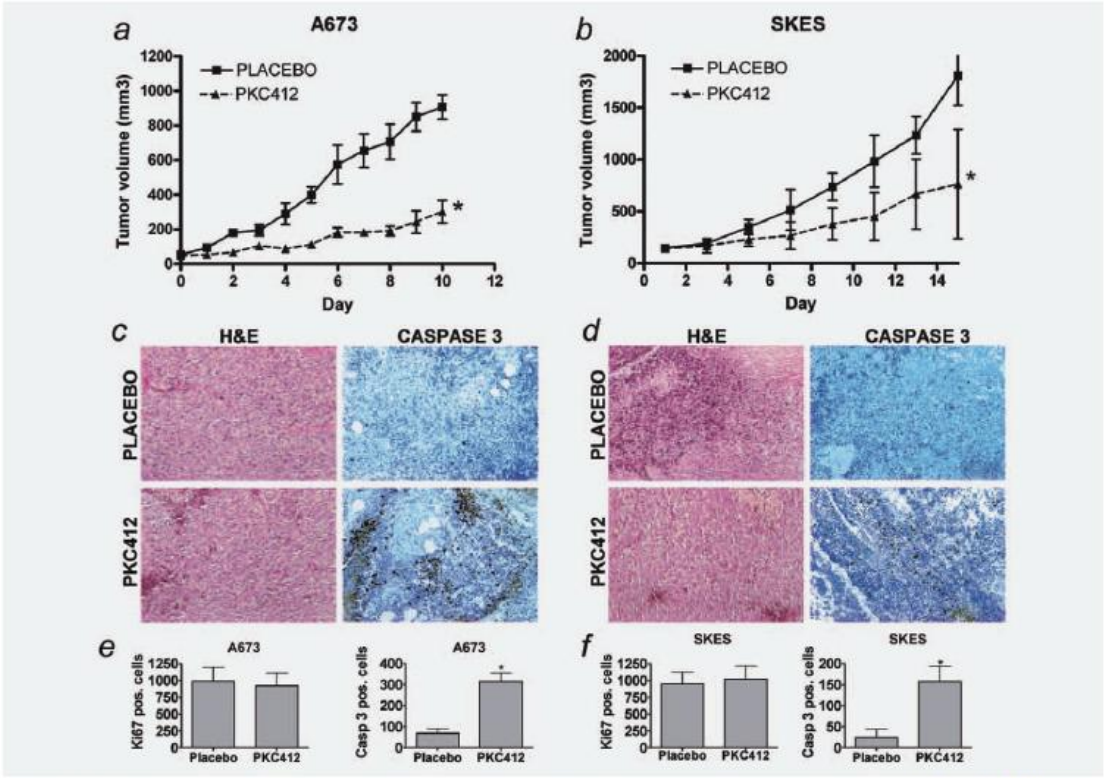


Figure 6. Inhibition of tumor growth by midostaurin in vivo. (*a*) Growth rate of A673 xenograft tumors in NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ mice treated with a daily dose of 100 mg/kg midostaurin (PKC412) for 10 days. (PKC412 $N = 5$ mice, placebo $N = 5$ mice). (*b*) Growth rate of SKES xenograft tumors in NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ mice treated every second day with a dose of 100 mg/kg midostaurin (PKC412) for 14 days. (PKC412 $N = 8$ mice, placebo $N = 8$ mice). (*c, d*) H&E and activated caspase 3 staining of A673 (*c*) and SKES (*d*) xenograft tumor sections treated with placebo or midostaurin. (*e, f*) quantification of Ki67 and activated caspase 3 positive cells counted using Image J software in A673 (*e*) and SKES (*f*) sections of tumors treated with placebo or midostaurin. Asterix indicates $p < 0.05$.

these xenograft models, tumor growth was significantly inhibited by midostaurin compared to placebo treatment (Figs. 6*a* and 6*b*, Supporting Information Fig. 1). To further characterize the effect of midostaurin *in vivo*, tumors were isolated, paraffin-embedded and morphology was studied by H&E staining. To quantify the influence of midostaurin on tumor-cell proliferation *in vivo*, tumor sections were immunohistochemically stained for the proliferation marker Ki-67 and activated caspase 3. As shown in Figures 6*c* and 6*d*, the number of apoptotic tumor cells was dramatically increased in tumor section of midostaurin-treated mice, whereas the number of Ki-67 positive cells was not changed (Supporting Information Fig. 2*a* and 2*b*), confirming the data obtained *in vitro*. Quantitative evaluation of these results is shown in Figures 6*e* and 6*f*. Interestingly, there was no significant influence of midostaurin on neoangiogenesis (Supporting Information Figs. 2*c* and 2*d*). These results suggest that midostaurin treatment leads to significantly reduced tumor growth *in vivo* mainly due to an increase in the number of apoptotic cells.

Discussion

Development of novel therapeutic approaches for pediatric sarcomas is a great challenge due to the rarity of these neoplasms. Although it has been recognized that expression of oncogenic fusion proteins generated by distinct chromosomal translocations is necessary for both tumor development as well as tumor maintenance, direct targeting of these transcription factors using small molecules is currently not possible. In this study, we therefore established an expression-based read-out system to measure modulation of transcriptional activity of EWS/FLI1 for small molecule discovery. This system was based on measuring expression levels of a few, well-characterized, direct target genes and was therefore an important simplification of the previously applied gene expression based high-throughput screening.[24] Nevertheless, the system represents a more

targeted approach than screenings based on survival or proliferation only and is more robust than a screening approach based on a single-gene reporter assay.[34] To further enhance the robustness of our approach, we quantitatively measured expression of genes that are both activated as well as repressed by EWS/FLI1 as we expected that unspecific killing would result in generally reduced mRNA levels.

Downregulation of EWS-ETS fusion proteins (EWS/FLI1, EWS/ERG, EWS/ETV1, EWS/ETV4, EWS/FEV and TLS/ERG) is associated with senescence or apoptosis.[35] Because the oncogenic role of EWS/FLI1 proteins in the Ewing family of tumors seems to result from abnormal regulation of transcription or RNA processing,[2,36–39] identification of target genes regulated by EWS/ETS proteins represents an important step in understanding the underlying molecular mechanisms. Hence, we combined microarray analysis of patient tumor biopsies with cell-line tumor models to define new EWS/FLI1 target genes using the following three different approaches: (i) we used data from three Ewing's sarcoma cell lines that were silenced for EWS/FLI1 with (ii) two different silencing methods (siRNA and shRNA) and (iii) compared these *in vitro* data to patient tumor biopsies. This resulted in a list of six genes that are candidate targets of the fusion protein (PTGER3, IL1RAP, DKK, CRIP1, ARHGAP and PHLDA1). The only gene in this list whose expression is low in Ewing's sarcoma patient tumors compared to other small blue cell tumors and is induced upon silencing of EWS/FLI1 was PHLDA1, suggesting that PHLDA1 is repressed by the fusion protein. Indeed, careful examination of previously published microarray data revealed PHLDA1 as top-ranking gene in two different studies as well,[9,26] thus confirming our analysis. In addition, we found low-PHLDA1 expression levels also in leukemia patients (data not shown) indicating that PHLDA1 might also be a target gene of FLI1 in a hematopoietic context. In support of this, it was shown that PHLDA1 expression can be regulated by another ETS family members, namely ETV6.[40] Moreover, PHLDA1 was shown to be downregulated during disease progression also in melanoma.[41] PHLDA1 is known to play a role in apoptosis regulation in mice,[42] and overexpression of PHLDA1 induces apoptosis in HUVEC cells.[43] Interestingly, preliminary data show that PHLDA1 overexpression has an inhibitory effect on cell proliferation and possibly on induction of apoptosis in Ewing's cells (AB, data not shown), supporting a possible role as a tumor suppressor.

From the 1,280 compounds screened with the small molecule library, we identified among the top 10 hits well-known chemotherapeutic agents such as camptothecin, etoposide, idarubicin and doxorubicin. Also included in this list was fenretinide, which is currently undergoing clinical trials for neuroblastoma and Ewing's sarcoma.[44,45] These results underscore the robustness of our screening approach and suggest that it can be expanded to additional compound libraries and collections of targeted agents in the future.

One of the most promising novel compounds identified in our screen was the pan-kinase inhibitor midostaurin. This is a small-molecule kinase inhibitor derived from staurosporine that inhibits several kinases, including protein kinase C, Akt/protein kinase B, c-Kit, FLT3 and fibroblast growth factor receptors.[46,47] Midostaurin is currently undergoing phase II clinical trials for the treatment of leukemias in adults and in children. As the population of patients available for phase I and II clinical trials with pediatric solid tumors is very limited, midostaurin represents a small molecule with already established low toxicity for such trials. In addition, midostaurin was already shown to be effective against a second pediatric sarcoma, rhabdomyosarcoma.[28] Hence, we further evaluated its effect against a large panel of Ewing's cell lines, which responded to midostaurin treatment at the high-nanomolarity range with massive apoptosis similar to etoposide currently used in Ewing's sarcoma therapy.

Importantly, this compound is also able to reduce cell growth, induce apoptosis and reduce tumor growth *in vivo*. Therefore, investigations toward the precise mechanism of action including identification of important kinases(s) in the EFT model are currently ongoing. Interestingly, Ewing's tumors are the second pediatric sarcoma after rhabdomyosarcoma that shows sensitivity toward treatment with the kinase inhibitor midostaurin. In rhabdomyosarcoma, midostaurin can directly modulate the phosphorylation status of the recurrent translocation PAX3/FKHR and thus inhibit its transcriptional activity.[48] Hence, we conclude that pan-kinase inhibitors could have a therapeutic potential in Ewing's sarcoma and possibly sarcomas in general. Therefore, they should be considered as effective agents alone or in combination with standard regimens for the future treatment of such childhood tumors.

Acknowledgements

We thank Michal Okoniewski for his precious comments on microarray analysis and Novartis Pharma AG for providing midostaurin. We also thank Valentina D'Alessandro for help with *in vivo* experiments. The animal experiments were approved by the local authorities (158/2009). The study was supported by grants from the Swiss National Science Foundation (31003A-118035) to F.N.

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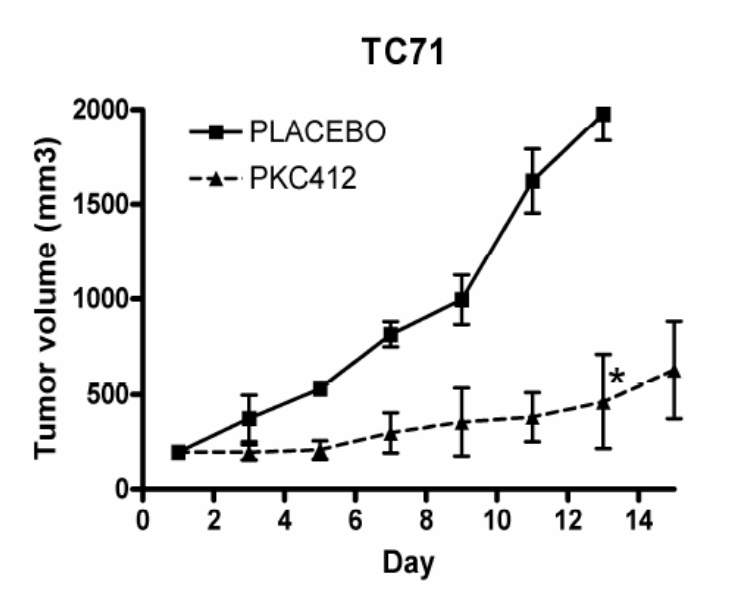
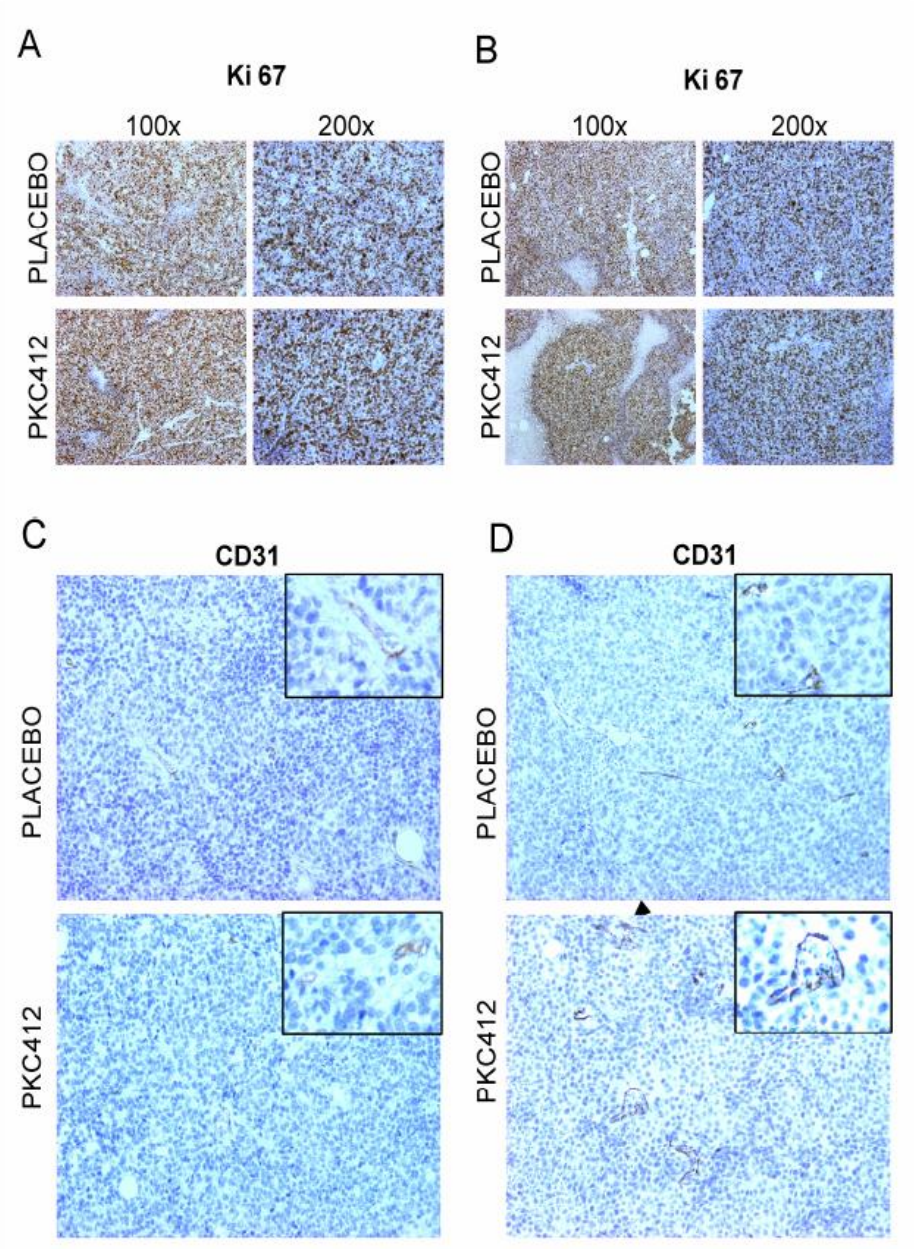


Supplementary Figure 1 Inhibition of tumor growth by midostaurin in vivo.
Growth rate of TC71 xenografts tumors in NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ mice treated every second day with a dose of 100 mg/kg midostaurin (PKC412) for 14 days. (PKC412 N=3 mice, placebo N=3 mice). Asterix indicates p≤0.05



Supplementary Figure 2 Midostaurin has no effect on proliferation and neoangiogenesis *in vivo*

Ki 67 staining of A673 (A) and SKES (B) xenograft tumor sections of placebo-treated and midostaurin (PKC412)-treated tumors. (C)(D) CD31 staining of A673 (C) and SKES (D) xenograft tumor sections of placebo-treated and midostaurin (PKC412)-treated tumors.

			Gene modulation in A673 cells*						
Compound*	Description	Company	EWS/FLI1	NR0B1	PHLDA1	Proliferation*	Class	Action	Selectivity
Stattic	STAT3 inhibitor	Sigma S7947	37	33	578	76	Gene Regulation	Inhibitor	STAT3
Mibefradil dihydrochloride	T-type Ca^{2+} channel blocker	Sigma M5441	27	25	225	57	Ca2+ Channel	Blocker	T-type
Fluphenazine dihydrochloride	Dopamine receptor antagonist	Sigma F4765	20	70	614	46	Dopamine	Antagonist	D1/D2
K114	Fluorescent amyloid-specific dye	Sigma K1015	40	18	215	70	Neurotransmission	Amyloid probe	
CGP-74514A hydrochloride	Cdk1 inhibitor	Sigma C3363	5	1	27	62	Phosphorylation	Inhibitor	Cdk1
Idarubicin	Antineoplastic/DNA Topoisomerase II inhibitor	Sigma I1656	16	5	8	52	DNA Metabolism	Inhibitor	
Sanguinarine chloride	Inhibitor of $Mg2+$ and $Na+/K+$-ATPase	Sigma S 5890	13	47	142	119	ion Pump	Inhibitor	Na+/K+ ATPase
Camptothecin	DNA topoisomerase I inhibitor	Sigma C9911	78	49	381	82	Apoptosis	Inhibitor	Topo I
Doxorubicin	Antineoplastic/DNA Topoisomerase II inhibitor	Sigma D1515	28	15	38	77	DNA Metabolism	Inhibitor	
Dipropylidopamine hydrobromide	Dopamine receptor agonist	Sigma D031	49	72	263	107	Dopamine	Agonist	
BIX 01294 trihydrochloride hydrate	Histone methyl transferase inhibitor	Sigma B9311	100	67	1261	74	Gene Regulation	Inhibitor	HMT
GR 127935 hydrochloride hydrate	5-HT1B/1D serotonin receptor antagonist	Sigma G5793	85	56	270	82	Serotonin	Antagonist	5-HT1B/1D
Retinoic acid p-hydroxyanilide	Antineoplastic Vitamin A acid analog	Sigma H7779	86	75	1019	58	Cell Cycle	Inhibitor	
beta-Lapachone	Anticancer agent	Sigma L2037	74	111	647	27	Apoptosis	Activator	
Methapyrilene hydrochloride	H1 Histamine receptor antagonist	Sigma M9125	81	31	187	117	Histamine	Antagonist	H1
Na-p-Tosyl-L-lysine chloromethyl ketone hydrochloride	Adenylyl cyclase inhibitor; NFkB inhibitor	Sigma T7254	47	82	198	109	Cyclic Nucleotides	Inhibitor	Adenylyl cyclase
PD 169316	p38 MAP kinase inhibitor	Sigma P9248	248	56	251	118	Phosphorylation	Inhibitor	p38 MAP kinase
2,2'-Bipyridyl	Metalloprotease inhibitor	Sigma B7505	31	74	130	112	Biochemistry	Inhibitor	Metalloprotease
Podophyllotoxin	Antineoplastic glucoside; inhibitor of microtubule assembly	Sigma P4405	77	90	349	56	Cytoskeleton and ECM	Inhibitor	
N-Oleoyldopamine	CB1 cannabinoid receptor ligand	Sigma O2139	75	96	533	107	Neurotransmission	Ligand	CB1
Retinoic acid	Caspase-dependent apoptosis inducer	Sigma R2625	69	93	220	120	Apoptosis	Activator	
Tyrphostin AG 835	Protein tyrosine kinase inhibitor	Sigma T5565	73	94	241	123	Phosphorylation	Inhibitor	Tyrosine kinase
Etoposide	Topoisomerase II inhibitor; induces apoptosis; cell cycle inhibitor	Sigma E1383	51	104	211	99	Apoptosis	Inhibitor	Topo II
alpha-Lipoic Acid	Coenzyme of pyruvate dehydrogenase and glycine decarboxylase	Sigma T5625	62	69	109	123	Cell Stress	Coenzyme	Pyruvate dehydrogenase
5-Bromo-2'-deoxyuridine	Thymidine analog; S-phase cell cycle inhibitor	Sigma B5002	105	51	109	116	DNA Metabolism	Inhibitor	
rac-2-Ethoxy-3-hexadecanamido-1-propylphosphocholine	PKC inhibitor	Sigma E3645	49	114	196	105	Phosphorylation	Inhibitor	PKC
Nefiracetam	Pyrrolidonetype nootropic agent	Sigma N2286	73	75	181	100	Neurotransmission	Nootropic	
Oxaspiol	Proapoptotic cell cycle inhibitor	Sigma G8761	106	70	202	110	Apoptosis	Inducer	
DOI hydrochloride	5-HT2 serotonin receptor agonist	Sigma D101	109	63	194	107	Serotonin	Agonist	5-HT2/5-HT1C
Palmitoyl-DL-Carnitine chloride	Modulator of PKC activation; intermediate in mitochondrial fatty acid oxidation	Sigma P4509	34	114	171	87	Phosphorylation	Modulator	PKC
Chelerythrine chloride	PKC translocation inhibitor	Sigma C2932	64	92	161	40	Phosphorylation	Inhibitor	PKC
PKC412	Multikinase inhibitor	Novartis	86	78	195	90	Phosphorylation	Inhibitor	

* Treatment: 5 µM of compound for 24 h
* In % of control (= 100%)
** p<0.05, unpaired two-tailed t-test, significant values written in bold

Supplementary Table 1 Selected Hits from LOPAC1280TM screening in A673 cells.
A673 cells were treated with compounds from the LOPAC1280TM library at a final concentration of 5 µM for 24 hours. Modulation of EWS/FLI1 transcriptional activity was monitored for expression of specified target genes NR0B1, PHLDA1 and for EWS/FLI1 itself. Cell proliferation measurement using WST-1 assay was performed in parallel. 32 compounds were selected for re-screening in additional Ewing sarcoma cell lines based on a 20% downmodulation of either EWS/FLI1 and/or NR0B1 and a 50% upregulation of PHLDA1. Relative gene expression levels are shown in percentage of solvent control (PBS/DMSO=100%).

5.2 Manuscript 2: PI3K/AKT pathway modulates transcriptional expression of EWS/FLI1

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Key words: Ewing's sarcoma, targeted inhibitor screening, PI3K signaling, EWS/FLI1 promoter.

Manuscript ready for submission.

Contribution: I performed screening together with FR, in vitro assays (Figure 2A) and promoter analysis (Figure 4). I further performed complete statistical analysis including screening as well as writing and formatting of this manuscript.

Abstract

Ewing sarcoma (ES) is a group of malignancies affecting bone and soft tissue in childhood and adolescence. The biology of these very aggressive osteolytic tumors revolves around EWS/FLI1, a chimeric transcription factor that is considered to be a hallmark of ES and represents an ultimate therapeutic target. Considering the difficulties in finding small molecule inhibitors for transcription factors, the window of opportunity lays in the understanding of the cellular processes affecting or being affected by the fusion protein and thus providing secondary targeting points. Therefore, in order to identify molecular pathway(s) that may contribute to the transcriptional activity and oncogenic properties of EWS/FLI1, we performed screening of a small library of 153 targeted inhibitors covering all major signaling pathways. A673 cells were treated with 500nM concentration of inhibitors for 24 hours. Having expression of EWS/FLI1 and its target genes PHLDA1, NROB1 and NKX2.2 as a read out, we discovered PI3K inhibitors as potent modulators of EWS/FLI1 expression. We confirmed this finding in four ES cell lines. To exclude the off target effect of the PI3K inhibitors we performed genetic loss of function experiments. Upon silencing catalytic subunits of PI3K we observed inactivation of the downstream signaling proteins followed by decrease of EWS/FLI1 on both mRNA and protein level. At the same time there was no effect of silencing PI3K on expression of FLI1 implying that the control of EWS/FLI1 expression is on the promoter level rather than at 3' UTR. Analysis of the EWS/FLI1 promoter region using various deletion constructs in luciferase assays determined two 12bp minimal elements where transcription factor(s) under PI3K control is binding. Elucidating the direct link between PI3K and EWS/FLI1 is of importance, and identity of the responsible transcription factor(s) might provide novel therapeutic opportunities.

Introduction

Ewing sarcoma (ES) is the second most frequent bone cancer in childhood. Clinically, ES appears as very aggressive osteolytic tumor with early tendency for development of metastasis. It belongs to the group of small-round-blue-cell tumors and is comprised of largely undifferentiated cells. The unique feature of this tumor is presence of the balanced t(11;22)(q24;q12) translocation in more than 85% of cases[1]. This gene rearrangement results in the expression of a chimeric fusion protein where RNA binding domain of EWS is exchanged by the DNA binding domain of the ets transcription factor FLI1, thus generating a dysregulated transcription factor EWS/FLI1[2]. More than 18 less represented alternative translocations involving EWS and other ets protein family members have been described since[3-8].

Extensive evidence supports the fact that EWS/FLI1 is an essential oncogenic component of ES development. Its oncogenic activity is thought to be mediated through inappropriate regulation of target genes that are crucial for the fully malignant phenotype[9-14]. Apart from having transforming and tumorigenic potential, even more important is that EWS/FLI1 appears to be necessary for tumor cell maintenance[15-19]. For these reasons EWS/FLI1 represents an attractive target. However, it is widely accepted that transcription factors are ‘’undruggable’’, and EWS/FLI1 as intrinsically disordered protein is not prone to direct inhibition by a small molecule in the classical sense.

No targeted agent has been routinely introduced into therapy of ES. Even though in the last few decades there has been considerable progress in both diagnosis as well as treatment of the localised disease, only 15% of patients with metastatic disease survive. Current treatment regimens of ES are not fully exploiting the fact that specific inhibition of signalling pathways is now possible. More specifically, it is not known whether specific pathway could affect the activity of EWS/FLI1.

Considering the difficulties in finding small molecule inhibitors for transcription factors, the window of opportunity lays in the understanding of the cellular processes affecting or being affected by the fusion protein and thus providing secondary targeting points. Therefore, in order to identify molecular pathway(s) that may contribute to the transcriptional activity and oncogenic properties of EWS/FLI1 we used a screening approach as previously published[20], and screened a small library that included a broad range of pathway inhibitors thus covering all major signalling

pathways. The aim of this study was to identify signalling pathways that are required for ES cell maintenance and thus provide novel indirect targeting options for EWS/FLI1.

Material and Methods

Cell lines

Three type 1 (A673, SKNMC, TC71) and two type 2 ES cell lines (SKES, RDES) were used. TC71 cells were kindly provided by Prof H. Kovar (St-Anna Children's Hospital, Vienna, Austria) and SKES and RDES by Prof. K.L. Schaefer (Institute of Pathology, Duesseldorf, Germany), A673 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultivated on 0.1% gelatine coated plates (Sigma-Aldrich, Buchs, Switzerland) in RPMI medium supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1% L-glutamine, at 37°C in 5% CO_2.

Screening assay

15 x 10^3 cells were plated in 96-well plates 24h prior to treatment. A library of 153 commercially available latest targeted inhibitors in the field of oncology was acquired from Axon Medchem and Selleck chemicals LLC. The complete list of compounds is shown in the Supplemental Table 1. Compounds were added to cells in complete RPMI medium at a final concentration of 500 nM for 24h. Lysis and subsequent cDNA synthesis was performed using Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies, #600559) according to the manufacturer's protocol, followed by quantitative PCR (qPCR) with corresponding target probes as described below. Similarly treated 96-well plates were used to measure cell viability in parallel using WST-1 cell proliferation kit (Roche Applied Sciences, Rotkreuz, Switzerland).

Quantitative PCR

Quantitative PCR (qPCR) was performed under universal cycling conditions on an ABI 7900 instrument using commercially available target probes and mastermix (all from Applied Biosystems, Rotkreuz, Switzerland). Data were analysed using SDS 2.2 software. CT values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression levels of the target genes were calculated using the $\Delta\Delta$CT method. All experiments were performed in

triplicate and repeated independently at least 3 times. Data analysis was done with the GraphPad prism software (San Diego, CA) and statistical analysis using the Student t-test. Commercially available target probes used were (Applied Biosystems): EFtype1:AIGI3UI, FLI1:Hs00956709_m1, PHLDA1:Hs00378285_g1, CAV1:Hs00184697_m1, NKX2.2:Hs00159616_m1, NR0B1: Hs03043658_m1, GAPDH:Hs99999905_m1, PIK3CA:Hs00907966_m1, PIK3CD:Hs00192399_m1, PIK3CG:Hs00277090_m1.

Silencing of PI3K

A total of 2 $x10^6$ A673 cells were seeded in a 60 mm Petri dish. On the same day, transfection was carried out using Lipofectamine RNAi MAX reagent (Invitrogen) and 5nM (final concentration) small interfering RNA (siRNA) of PI3K Cα (Ambion s10520), PI3K Cδ (Ambion s10530), PI3K Cγ (Ambion s10532). As a negative control scrambled siRNA no. 1 (Quiagen S103650318) was used. Cells were lysed 48 hours after silencing and subsequent RNA extraction using RNA easy mini kit (Quiagen) was performed followed by cDNA synthesis with RT kit (Applied Biosystems).

Immunoblotting

Cells were washed twice with PBS and harvested in lysis buffer containing 50mM NaH_2PO_4 (pH=7.5), 150mMNaCl, 1% Triton X-100, 1mM Na3OV4, 5mM Na-pyrophosphate, 40nM NaF, 1mM EGTA supplemented with protease inhibitor cocktail (Complete + 1mM EDTA, Roche). Protein concentration was determined by the method of Bradford (Biorad, Reinach, Switzerland). 10–30µg of protein extract was resolved on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Whatman, Germany). Primary antibodies were used as follows: anti-FLI1 monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, USA) (dilution 1:1000), anti-PARP rabbit polyclonal antibody (Cell Signalling Technology, Berverly, MA, USA) (dilution 1:1000), anti-pAKT antibody (Ser473, Cell Signalling) (dilution 1:1000), anti-AKT antibody (Cell Signalling) (dilution 1:1000), anti-PHLDA1 antibody (Sigma Aldrich)(1:1000), anti-phospho-mTOR antibody (Ser2448, Cell Signalling) (1:1000), anti-mTOR antibody (Cell Signalling) (1:1000), anti-phospho-S6 Ribosomal protein antibody (Ser235/236, Cell Signalling) (1:2000),anti-S6 Ribosomal protein antibody (Cell Signalling) (1:1000) and anti-β-tubulin I mouse monoclonal antibody (Sigma-Aldrich) (dilution 1:40’000). After incubation with the

appropriate secondary peroxidase-conjugated antibodies (1:1'000), detection was performed with the ECL chemiluminescent reagent (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions.

Luciferase assays

2.3kb of the promoter region of EWS/FLI1 (Ref. sequence NM_013986) (position -2239/+80 relative to the transcription initiation site) was cloned into pGL4.19 luciferase vector (Promega) using the In Fusion HD Cloning Kit (Clontech Laboratories Inc.). Using the same approach several deletion constructs of the EWS/FLI1 promoter were constructed, namely -1708/+80, -1277/+80, -774/+80, -275/+80 (position relative to the transcription initiation site). Using Site directed mutagenesis kit (Invitrogen) additional 24 deletion mutants of the -2239/+80 plasmid were made (detailed list of the plasmids with description of the mutations is in the Supplemental Table 2).

$2x10^3$ A673 cells per well were plated in 96-well plate and 24h later transfected using Jet Prime (Polyplus Transfection) with 100ng of reporter construct, or empty vector (pGL4.19) as a negative control. The cells were also transfected with 10ng of Renilla luciferase plasmid for normalization of transfection efficiency. After 24h cells were treated with 50nM BEZ235 or DMSO. 48h after transfection cells were lysed and assayed for luciferase activity using the Dual Glo luciferase reporter system (Promega) according to manufacturer's instructions.

Results

Screening a small library of targeted inhibitors identifies PI3K pathway as modulator of EWS/FLI1 activity

To identify molecular pathway(s) that might contribute to transcriptional activity and oncogenic properties of EWS/FLI1, we used our previously established and validated screening protocol[20] to screen a second library of targeted inhibitors against a variety of molecular pathways. The collection of 153 inhibitors (Supplemental Table 1) was screened for EWS/FLI1 target gene modulation as primary read out in A673 ES cells at a final concentration of 500 nM in duplicates. Modulation of EWS/FLI1 transcriptional activity was monitored via expression of the specific target genes NR0B1[21], PHLDA1[20], NKX2.2[22] and complemented by measuring EWS/FLI1 levels

itself. Since PHLDA1 is repressed and NROB1 and NKX2.2 are activated target genes of EWS/FLI1, inhibition of EWS/FLI1 activity is expected to result in up regulation of PHLDA1 and down regulation of NROB1 and NKX2.2, independent of direct or indirect effects. Cytotoxicity measurement in duplicates using WST-1 assays was performed in parallel. The final hit-list is based on a significant ($p<0.05$, unpaired two-tailed t-test) modulation of at least two out of three target genes compared to the untreated controls in A673 cells. The results are summarized in the Figure 1 for the top 16 inhibitors. We identified inhibitors targeting several signalling pathways, both known and unknown to play a role in sarcomas. The most prominent among them is the phosphoinositide-3-kinase (PI3K) pathway, which is targeted by three different inhibitors. Inhibition of this pathway provoked a significant modulation of EWS/FLI1 target genes and a strong inhibition of cell proliferation in A673 ES cell line. Hence, these experiments identified PI3K signalling to modulate expression of EWS/FLI1 target genes.

			EWS/FLI1 target gene modulation in A673 cells*,**					
Compound[a]	Target	Company	EWS/FLI1	PHLDA1	NR0B1	NKX2.2	Nr. of sign. target gene response**	Proliferation*
NVP-BEZ235	PI3K/mTOR inhibitor	Axon 1281	89	**232**	**65**	**40**	3	58
PIK 75	PI3K/p110 alpha inhibitor	Axon 1334	**8**	115	**9**	**5**	2	54
NVP-BAG956	PI3K/PDK1 inhibitor	Axon 1282	111	**176**	83	**68**	2	68
DBZ	Gamma Secretase inhibitor	Axon 1488	116	**183**	**85**	**77**	3	113
BZ	Gamma Secretase inhibitor	Axon 1487	102	**181**	88	**68**	2	115
Vorinostat	HDAC inhibitor	Cayman	**47**	**185**	**61**	**68**	3	124
Bosutinib (SKI 606)	BCR-ABL/SRC inhibitor	Axon 1407	92	**221**	**84**	**56**	3	121
Tacrolimus	Calcineurin inhibitor	Axxora	62	**126**	**71**	**67**	3	106
YM155	Survivin inhibitor	Selleck 1130	**25**	120	**28**	**46**	2	2
LY 2157299	TGF beta inhibitor	Axon 1491	73	153	**76**	**68**	2	108
Velcade	Proteasome inhibitor	Cilag	77	119	**17**	**18**	2	45
ICG-001	CBP/Beta-Catenin inhibitor	Michael Kahn	61	109	**72**	**69**	2	100
GDC-0449	Hedgehog Pathway Inhibitor	Selleck 1082	97	147	**86**	**63**	2	114
Tandutinib	FLT3 inhibitor	Axon 1415	61	**137**	105	**66**	2	103
TG101348	JAK2 inhibitor	Symansis	**59**	**128**	97	**77**	2	100
NU 1025	PARP inhibitor	Axon 1370	90	**130**	87	**81**	2	120

[a] Treatment: 500 nM of compound for 24 h
* in % of control (=100%)
** p<0.05, unpaired two-tailed t-test, significant values written in bold

Figure 1 ***Screening a small library of targeted inhibitors identifies PI3K pathway as modulator of EWS/FLI1.*** Relative mRNA expression levels of EWS/FLI1 and target genes NR0B1, PHLDA1 and NKX2.2 in A673 cells after 24h treatment with 500 nM of indicated compounds were measured by quantitative RT-PCR. Cell proliferation measurement using WST-1 assay was performed in parallel. Values are shown in percentage of untreated control (=100%) and represent mean of 2-4 independent experiments performed in duplicate. Significant values are written in bold ($p<0.05$, unpaired two-tailed student t-test).

BEZ235 treatment alters EWS/FLI1 expression

Since BEZ235, a dual inhibitor of PI3K and the downstream mammalian target of rapamycin (mTOR) with well described mode of action, was the only PI3K inhibitor to significantly

modulate all three EWS/FLI1 target genes, we validated the importance of this pathway further using this compound. Since our screening does not allow to discriminate between compounds with effects on activity or expression of EWS/FLI1, we addressed this question first. Upon treatment of four ES cell lines with 500nM BEZ235 we observed decrease to less than 50% of EWS/FLI1 expression on mRNA level (Figure 2A). This decrease of EWS/FLI1 levels was followed by corresponding response of its target genes, namely NKX2.2, NROB1 and PHLDA1. The effect of BEZ235 on EWS/FLI1 was confirmed also on protein level (Figure 2B) with a clear dose dependency. This also corresponded with induction of apoptosis, as measured by PARP cleavage. Hence, this data suggest that inhibition of PI3K signalling reduces expression of EWSFLI1 on both RNA and protein level.

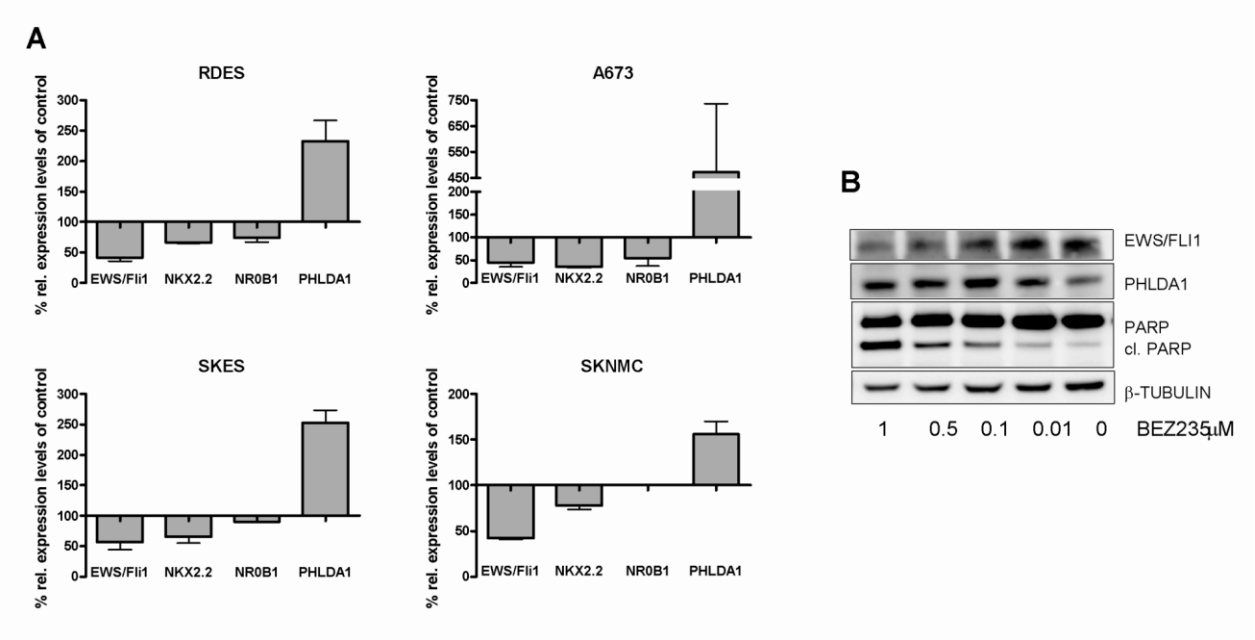

Figure 2 BEZ235 affects EWS/FLI1 levels (A) Relative expression of EWS/FLI1 and its target genes measured by quantitative RT-PCR upon 24h treatment with 500nM BEZ235 **(B)** Protein expression measured by western blot of EWS/FLI1, PHLDA1, PARP and β-tubulin as loading control. Cells were treated for 24h with indicated concentrations of BEZ235.

Silencing PI3K pathway has effect on EWS/FLI1 expression

Treatment with BEZ235 revealed significant changes in PHLDA1, NR0B1 and NKX2.2 expression on the mRNA level. To exclude unspecific off-target effects of the drug we performed

genetic-loss of function experiments using siRNA targeting the catalytic domains α, δ and γ of class I PI3Ks in A673 cells. Treatment for 48 hours resulted in down regulation of PI3KC α, δ and γ mRNA by 75% as measured by quantitative RT-PCR (Figure 3A), compared to scrambled control. In PI3K silenced cells PHLDA1 showed a 10 fold up regulation, whereas target genes NR0B1, NKX2.2 and CAV1 were down regulated by 70%, 55% and 45%, respectively (Figure 3B). Notably, expression of EWS/FLI1 was down regulated by 65%, as well only a minor effect was observed for FLI1 (7% down regulation), which was used as negative control to exclude a general impact of BEZ235 on gene expression. These results could be confirmed by western-blot analysis. Silencing of PI3K pathway also led to inhibition of the downstream effectors AKT, mTOR and S6 ribosomal protein as shown by phospho-specific antibodies and as expected (Figure 3C). Altogether, silencing of PI3Ks elicited the same effect on expression of EWS/FLI1 and its target genes as did treatment with BEZ235 thereby validating the use of BEZ235 as specific inhibitor in further experiments.

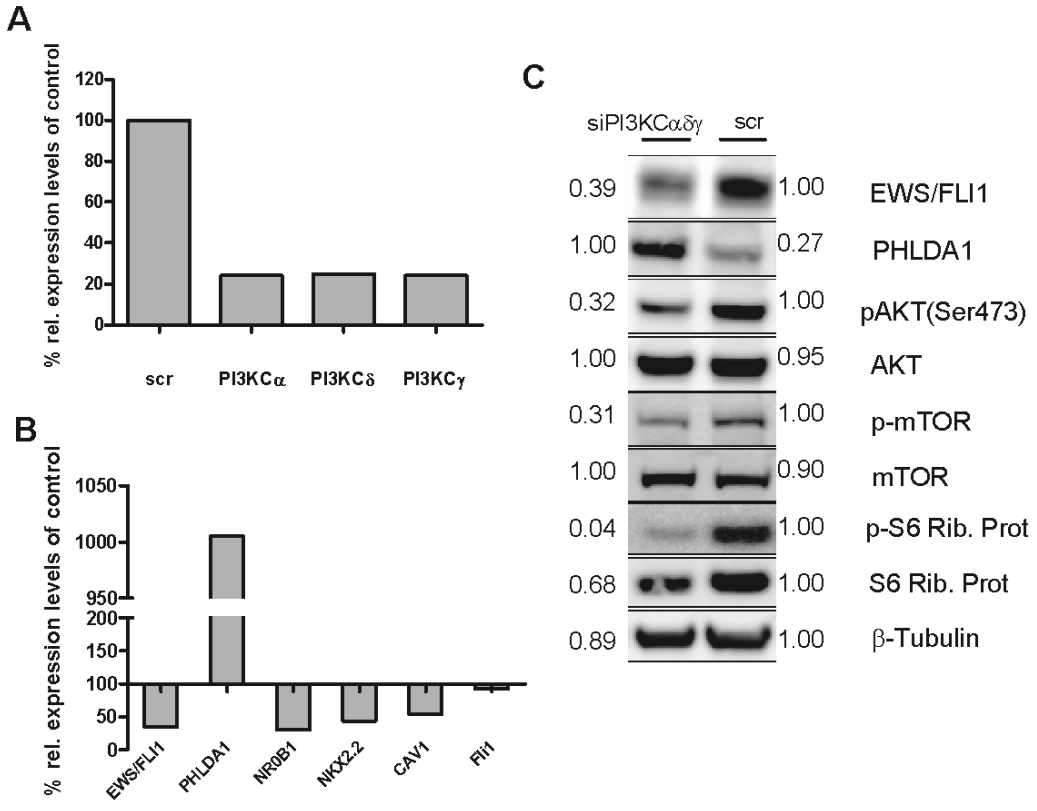

Figure 3 Modulation of EWS/FLI1 and target genes after PI3K pathway silencing. (A) PI3KC α,δ and γ mRNA expression levels were measured in A673 cells after silencing for 48h compared to scrambled control by qRT-PCR. **(B)** EWS/FLI1 and its target genes mRNA expression upon silencing of PI3KC α, δ and γ in A673 cells for 48 hours.

(C) EWS/FLI1, PHLDA1 and PI3K downstream effectors expression levels after silencing in A673 cells for 48 hours (numbers on the left and right side of the blot represent intensity of bands measured by densitometry)

PI3K signalling regulates EWS/FLI1 expression on its promoter level

Considering that FLI1 levels did not change upon inhibiting the PI3K pathway while at the same time EWS/FLI1 expression was reduced, we hypothesized that regulation of EWS/FLI1 occurs on the promoter level. To test this we performed a luciferase assay with a plasmid containing the 2.3kb promoter of EWS in front of the reporter. Since BEZ235 has strong pro apoptotic effect on ES cells with IC50 at 498nM (Supplemental Figure 1), we first determined the minimal concentration capable to elicit a 2 fold decrease in luciferase activity as seen with endogenous EWS/FLI1 levels. A673 ES cells were transfected with 2.3kb promoter construct and concentration of BEZ235 titrated for 24h (Figure 4A). With increasing concentration of BEZ235 we observed dose dependent decrease in the luciferase activity. At the same time, already 50nM BEZ235 treatment was able to reduce luciferase activity by 50% which was used for further experiments. Nevertheless, these experiments imply that indeed this promoter region contains a regulatory element responsive to PI3K signalling. To narrow down the region of interest we designed several additional deletion constructs. The results show that the regulatory element is still present within the smallest deletion construct (-275bp relative to the transcription initiation site). To exclude possible effects of BEZ235 on the stability of luciferase itself, cells were also transfected with a constitutive promoter of luciferase. Evidently, the compound did not interfere with luciferase activity (Figure 4B).

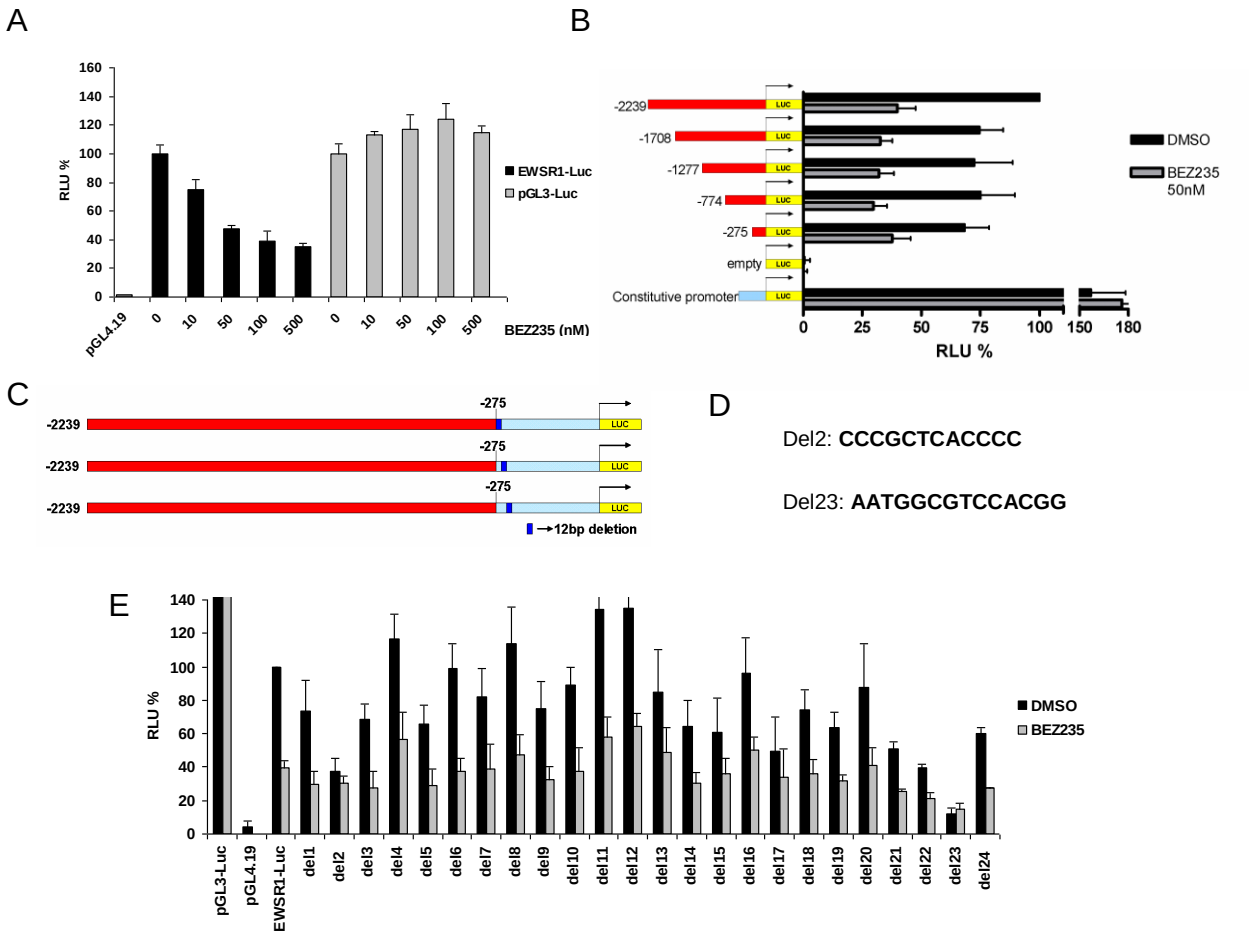

Figure 4 EWS/FLI1 promoter analysis by luciferase assay (A) Relative luciferase activity in A673 cells transfected with different constructs (pGL4.19-empty vector control, pGL3- constitutive promoter control, EWSR1-Luc- 2.3kb promoter vector) and treated for 24h with different concentration of BEZ235 **(B)** Relative luciferase activity in A673 cells transfected with different deletion constructs of EWS/FLI1 promoter. Cells were treated with either DMSO or 50nM BEZ235 **(C)** Scheme of the cloning process for making sequential 12bp deletion constructs from the 2.3kb promoter construct **(D)** Luciferase assay performed as above, with a series of deletion constructs of the 2.3kb EWS/FLI1 promoter **(E)** Sequences of the two minimal binding elements that are absent from the deletion constructs Del2 and Del23.

In order to pinpoint further the regulatory element within -275/+80 region of the promoter, we designed a series of deletion constructs where 12-14bp were deleted from the full size promoter (Figure 4C). If the regulatory element is excised from the promoter we expect that control by PI3K pathway is lost and hence there should be no difference in luciferase activity upon treatment with BEZ235. Out of 24 constructs only two, construct number 2 and construct number 23 were no more affected by the treatment with BEZ235, implying that the PI3K regulatory elements are within these two 12bp sequences (Figure 4D and Supplementary table

2). The sequence of these elements that contain the responsive element is shown in Figure 4E. In summary, our results suggest that inhibition of PI3K pathway, through control of a transcription factor(s) that binds in a 12bp minimal element, can interfere with expression of EWS/FLI1 on the mRNA level.

Discussion

Despite increasing efforts there is still no successful targeted therapy for ES. Recently, several novel targeted approaches have been initiated in clinical trials[23-27]. However, it is clear that complexity of ES is not caused by a single driving event and therefore it is necessary to elucidate all the major contributors to the fully malignant phenotype. This approach should offer possibilities for several therapeutic targets and in combination therapy it should be possible to circumvent potential resistance to any given single drug.

Here, we conducted a screening of a small library of targeted inhibitors that are inhibiting different signaling pathways in order to identify critical pathways. We employed our well established screening approach that uses expression of three EWS/FLI1 target genes as surrogate markers of EWS/FLI1 activity[20] and was therefore an important simplification of the previously applied gene expression based high-throughput screening[28]. Nevertheless, the system represents a more targeted approach than screenings based on survival or proliferation only, and is more robust than a screening approach based on a single-gene reporter assay[29]. Given the fact that EWS/FLI1 expression is crucial for tumor cell maintenance, diminished activity/expression should also result in apoptosis and reduced cell proliferation[15-19]. As the most prominent pathway that regulates EWS/FLI1 activity/expression we identified the PI3K. This corroborates earlier findings demonstrating that IGF1R signaling via PI3K is a very important axis in ES cells[30-33]. Indeed, inhibition of IGF1R using monoclonal antibodies showed good initial response in a subgroup of patients. IGF1R activates several signaling pathways, whereas MAPK pathway is crucial for cell proliferation and PI3K pathway for anti-apoptotic effect[34]. Combined therapy of anti IGF1R antibodies and PI3K/mTOR inhibitors showed improved response. Nevertheless, even in these patients resistance was observed. This implies that alternative pathways are taking over thus providing tumor cell survival.

Using BEZ235, a dual PI3K and mTOR inhibitor, as the most potent compound in our hit list we observed a strong decrease in EWS/FLI1 on both RNA and protein level. Surprisingly, very little

is known about the regulation of EWS/FLI1 expression. So far, only one study is available suggesting possible regulatory transcription factors[35]. However, it has been shown previously that inhibition of mTOR by rapamycin decreases EWS/FLI1 protein levels[36]. Therefore, we validated BEZ235 with genetic-loss of function experiments in order to exclude unspecific off-target effects. These experiments demonstrated that the effect on the EWS/FLI1 expression is specific. Moreover, by performing a series of luciferase experiments with the EWS/FLI1 promoter we showed for the first time the direct control of EWS/FLI1 promoter and thus its expression by the PI3K pathway. Further on, we were able to determine the exact regulatory element within the promoter. Additional studies are now necessary to determine the identity of the transcription factor(s) under PI3K control that is binding in this region. Computational analysis of the sequence using weight matrices implies several potential binding factors, both known as well as unknown to be controlled by the PI3K pathway. One of the candidates is NF-κB whose activity and intracellular localization is known to be controlled by PI3K signaling. However, further experimental confirmation is needed.

Targeted inhibition of IGF1R/PI3K signaling shows very promising results for treatment of ES[23, 25-26, 37]. However, we still do not know the exact mechanisms responsible for this efficacy. It seems there is a need for more direct targeting of EWS/FLI1 since it appears that silencing EWS/FLI1 has stronger pro apoptotic effect than IGF1R antibody[38]. One possible explanation is that IGFBP3 as target of EWS/FLI1 can induce apoptosis independently of IGF system[39]. Also, there are other EWS/FLI1 target genes that might contribute to induction of apoptosis upon silencing EWS/FLI1. Considering all this, it is possible that apoptosis induced by BEZ235 or other IGF1R/PI3K inhibitors is at least partially a result of decrease in EWS/FLI1 expression. Therefore, elucidating the direct link between PI3K and EWS/FLI1 is of importance, and identity of the responsible transcription factor(s) might provide novel therapeutic opportunities.

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Supplementary Data

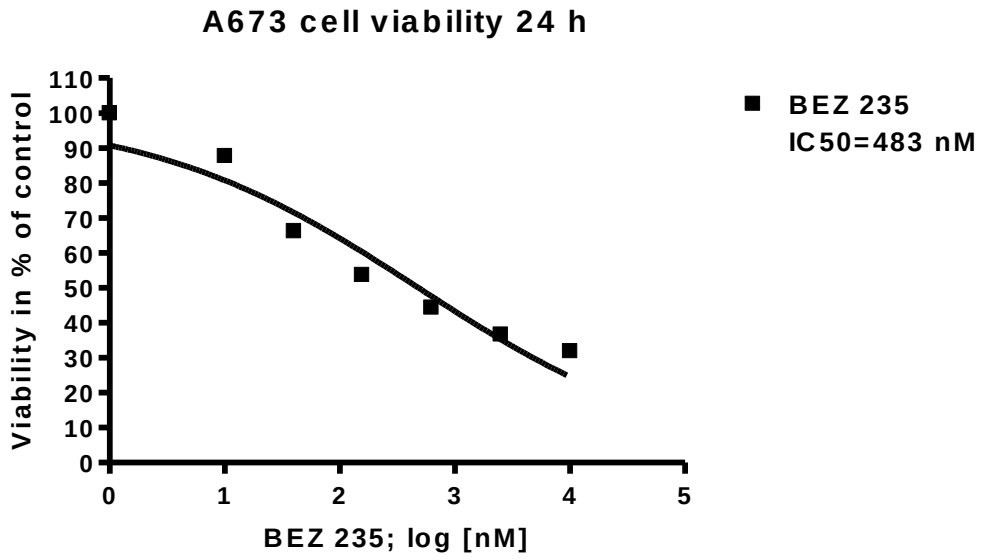
Supplemental Table 1: List of targeted inhibitors used in the screening

No.	Name	Target
1	[Ala92]-p16 (84-103)	Cdk inhibitor
2	17-AAG	Hsp90 inhibitor
3	2-Deoxy-D-glucose	Glycolysis inhibitor
4	3-MA	Vps34 inhibitor (class III PI3K)
5	A 769662	AMPK activator
6	ABT-102	TRVP1 antagonist
7	ABT-737	BCL-2, BCL-xl inhibitor
8	AEG 3482	JNK inhibitor
9	AG 013736 - Axitinib	VEGFR inhibitor
10	AMD3100	CXCR4 inhibitor
11	AMN 107 (Nilotinib)	BCR-ABL inhibitor
12	Apratastat	TACE/MMP inhibitor
13	AS 252424	PI3K p110 gamma inhibitor
14	AT9283	Aurora kinases Inhibitor
15	Atazanivir	Protease inhibitor
16	AZD 2281 - Olaparib	PARP inhibitor
17	AZD 7762	CHK inhibitor
18	AZD1152-HQPA	Aurora B inhibitor
19	BACE 1 inhibitor	Beta Secretase inhibitor
20	Bax inhibitor peptide P5	Bcl-2 Protein Family Inhibitor
21	Bax inhibitor peptide V5	Bcl-2 Protein Family Inhibitor
22	Bax inhibitor peptide,negative control	Bcl-2 Protein Family Inhibitor
23	BAY 61-3606	Syk inhibitor
24	BI 2536	PLK-1 inhibitor
25	BMS 189961	RAR gamma agonist
26	BMS 270394	RAR gamma agonist
27	BMS-345541	IκB inhibitor
28	Bosutinib (SKI 606)	BCR-ABL/SRC inhibitor
29	Butabindide	TPPII inhibitor
30	BX 795	PDK1/TBK1 inhibitor
31	BX 912	PDK1 inhibitor
32	BZ - Gamma Secretase inhibitor BZ	Gamma Secretase inhibitor
33	Cediranib	VEGFR inhibitor
34	CH 55	RAR alpha/beta agonist
35	Chir98014	GSK-3 inhibitor
36	CI-1033 - Canertinib	EGFR inhibitor
37	Combretastatin-A4 (CA-4)	Inhibitor of tubulin polymerization
38	Compound C	AMPK inhibitor
39	CP 690550	JAK3 inhibitor
40	CT 99021 - CHIR 99021	GSK-3 inhibitor
41	Cyclopamine	Hedgehog Pathway Inhibitor
42	Cyclosporine	Immunosupressant
43	DAPT	Gamma Secretase inhibitor
44	Dasatinib	BCR-ABL/SRC inhibitor
45	DBZ	Gamma Secretase inhibitor
46	Deguelin	Anticancer agent
47	DM 3189	BMP inhibitor
48	Doramapimod	p38 MAPK inhibitor
49	DR 2313	PARP inhibitor
50	FK 866	NAPRT1 inhibitor, anti-cancer agent
51	GDC 0879	B-Raf inhibitor
52	GDC 0941	PI3K inhibitor
53	GDC-0449	Hedgehog Pathway Inhibitor
54	Gefitinib	EGFR inhibitor
55	GSK 269962A	ROCK1 inhibitor
56	GW 441756	TrkA inhibitor
57	GW 786034	VEGFR/KIT/PDGFR inhibitor
58	GW 843682X	PLK inhibitor
59	Honokiol	pAkt, scr, p44/42 MAPK
60	HU-308	CB2 agonist
61	IGC-001	Beta-Catenin inhibitor
62	Imatinib	BCR-ABL inhibitor
63	JAK inhibitor I	JAKs inhibitor
64	JIP-1 (153-163)	JNK Inhibitor
65	JWH 018	CB2 agonist

66	JWH 073	CB1/2 agonist
67	JWH 133	CB2 agonist
68	Ko 143	BCRP inhibitor
69	KU-55933	ATM inhibitor
70	L-685,485	Gamma Secretase inhibitor
71	L-aminoadipic acid	Glutamine synthase inhibitor
72	Lamotrigine	Glutamate antagonist
73	Lapatinib	EGFR/ErbB-2 inhibitor
74	LE-135	RAR beta antagonist
75	LY 2157299	TGF beta receptor inhibitor
76	LY 294002	PI3K inhibitor
77	Masitinib mesylate	KIT/PDGFR inhibitor
78	MK 1775	Wee1 inhibitor
79	MK-2206	Akt inhibitor
80	MLN8237	Aurora kinase inhibitor
81	Myoseverin	Microtubule inhibitor
82	Na-Butyrate	HDAC inhibitor
83	NEC-1	Necroptosis/RIPK inhibitor
84	NF-kB Inhibitor	NF-kB Inhibitor
85	Nocodazole	Cell cycle G2/M inhibitor
86	NSC 348884	Nucleophosmin inhibitor
87	NSC 625987	CDK4 inhibitor
88	NU 1025	PARP inhibitor
89	NU 7441	DNA-PK inhibitor
90	NVP-AEW514	IGF-1R inhibitor
91	NVP-AUY922	HSP90 inhibitor
92	NVP-BAG956	PI3K/PDK1 inhibitor
93	NVP-BEZ235	PI3K/mTOC inhibitor
94	NVP-BGJ398	FGF-R inhibitor
95	NVP-BKM120	PI3K, not mTOC inhibitor
96	NVP-BSK805	JAK2 inhibitor
97	NVP-TAE684	ALK inhibitor
98	NVP-TKI258	FGF-R inhibitor
99	Obatoclax	Bcl-2 inhibitor
100	OSI 774 - Erlotininb	EGFR inhibitor
101	OSI-027	mTORC1/2 inhibitor
102	Palmitoylethanolamide	Endocannabinoid
103	PD 0325901	MEK inhibitor
104	PD 166793	MMP inhibitor
105	PD 169316	p38 MAPK inhibitor
106	PD 180970	Src kinase inhibitor
107	PD 184352	MEK 1 inhibitor
108	PD 98059	MEK inhibitor
109	PD150606	Calpain inhibitor
110	PF-00356231	MMP-12 inhibitor
111	PHA-739358(Danusertib)	Aurora kinases,Bcr-Abl and FGFR inhibitor
112	PI 103	Class I PI3K inhibitor
113	piceatannol	Syk inhibitor
114	PIK 75	PI3K p110 alpha inhibitor
115	PIK 90	PI3K p110 alpha inhibitor
116	pimecrolimus	Calcineurin inhibitor
117	PLX 4720	B-Raf inhibitor
118	PP2	Src inhibitor
119	PP242	mTORC1/2 inhibitor
120	PU-H71	HSP90 inhibitor
121	Roscovitine/Celiciblib	Cdk inhibitor
122	Ruboxistaurin (LY333531)	PKC beta inhibitor
123	S31-201	Stat3 Inhibitor
124	Saracatinib	Src and Abl inhibitor
125	SB 202190	p38 MAPK inhibitor
126	SB 203580	p38 MAPK inhibitor
127	SB 216763	GSK-3 inhibitor
128	SB 431542	TGF beta receptor inhibitor
129	Scriptaid	HDAC inhibitor
130	SD 169	p38 MAPK inhibitor
131	SD 208	TGF-betaR receptor 1 inhibitor
132	SL 327	MEK1/2 inhibitor
133	SNS-314	Aurora kinase inhibitor
134	Sorafenib (BAY 43-9006)	Raf/Mek/Erk inhibitor
135	Stobadine	Antioxidant
136	SU 6656	Src kinase inhibitor
137	SU11274	c-Met inhibitor
138	Sunitinib - SU 11248	Multiple RTK inhibitor
139	Tacrolimus	Calcineurin inhibitor
140	Tandutinib	FLT3 inhibitor
141	TG101348	JAK2 (Flt3) inhibitor
142	TGX 221	PI3K p110 beta inhibitor
143	Tiplaxtinin	PAI-1 inhibitor
144	TW-37	Bcl-2 protein family inhibitor
145	Tyrphostin AG 490	JAK2 inhibitor
146	U 73122	Phospholipase C inhibitor
147	Vandetanib	VEGFR/EGFR Inhibitor
148	Velcade	Proteasome inhibitor
149	Vorinostat/SAHA	HDAC inhibitor
150	VPA	HDAC inhibitor
151	xav-939	Wnt/beta-catenin signal transd. inhibitor
152	XL228	Multiple Tyr-kinase inhib. (IGF1-R, Bcr-Abl)
153	Y-27632	p160 ROCK inhibitor

Supplemental Table 2: Deletion constructs of the 2.3kb EWS/FLI1 promoter construct

Name of the construct	Position of the deletion relative to the transcription initiation site
Del1	-214/-203
Del2	-202/-191
Del3	-190/-179
Del4	-178/-167
Del5	-166/-155
Del6	-154/-143
Del7	-142/-131
Del8	-130/-119
Del9	-118/-107
Del10	-106/-95
Del11	-94/-83
Del12	-82/-71
Del13	-70/-59
Del14	-58/-47
Del15	-46/-35
Del16	-34/-23
Del17	-22/-11
Del18	-10/+2
Del19	+3/+14
Del20	+15/+26
Del21	+27/+40
Del22	+41/+54
Del23	+55/+68
Del24	+69/+80



Supplemental Figure 1 A673 ES cells viability after 24h treatment with titrating concentration of BEZ235

5.3 Additional studies: Functional relevance of PHLDA1 repression in Ewing sarcoma

Introduction

PHLDA1 (Plekstrin homology-like domain, family A, member 1), also known as TDAG51 (T-cell death-associated gene 51) belongs to a family of proteins (together with PHLDA2 and PHLDA3) and they all have in common a motif that resembles pleckstrin homology (PH) domain. Despite the fact that proteins with pleckstrin homology-like domains are often involved in different cellular processes such as cell adhesion, modulation of cell morphology or apoptosis, the function of PHLDA1 is still not fully elucidated. For the first time PHLDA1 was described in mouse T cell hybridoma where stimulation with antigen or an anti-T cell receptor antibody induced production of IL-2[222]. At the same time, mouse T cell hybridoma underwent Fas-FasL interaction dependent apoptosis. It was showed that PHLDA1 is required for mouse Fas (CD95) expression[222]. However, in humans this mechanism was not confirmed. When constitutively expressed in various cellular models of PHLDA1 is associated with an increase of basal apoptosis, reduced cell growth, cloning efficiency and colony formation[223-226]. PHLDA1 is hypothesized to play a role in inhibition of translation[227] and it binds proteins that are involved in RNA processing like lL14, eIF3-p66 or inducible polyA binding protein (iPABP).

The role of PHLDA1 in tumors became the topic in recent time. There are few studies implying an oncogenic activity of PHLDA1[228-229]. PHLDA1 is a putative epithelial stem cell marker in the human small and large intestine and contributes to migration and proliferation in colon cancer cells[230]. However, there is even more evidence that it acts as a tumor suppressor in certain tumor types[226, 231]. In human melanoma cells, when PHLDA1 is constitutively expressed it enhances the sensitivity to the chemotherapeutic agents doxorubicin and camptothecin[226]. In breast cancer PHLDA1 was shown to be a strong prognostic marker, where down regulation is being connected to poor survival[231-232]. PHLDA1 is crucial negative regulator of Aurora A kinase in breast cancer and at the same time is its substrate[233].

Previously we described PHLDA1 as a target gene of EWS/FLI1 in ES[234]. Through direct binding at the promoter of PHLDA1, EWS/FLI1 represses its expression. It is believed that through deregulated expression of many target genes EWS/FLI1 “drives” the fully malignant phenotype of ES cells[65, 99-102, 235]. Out of these target genes, quite many up-regulated target genes have been functionally characterized but only a hand full of directly repressed genes. Given the

fact that PHLDA1 is repressed in ES cells, we hypothesized that there must be some selective advantage for cancer cell and in that light PHLDA1 would probably have a tumor suppressor role rather than oncogene. Here we investigated the role of PHLDA1 in proliferation, apoptosis, cell detachment and cell adhesion in ES.

Material and Methods

Cell lines and plasmids

RDES (type II fusion) cells were kindly provided by Prof. K.L.Schaefer (Institute of Pathology, Duesseldorf, Germany), A673 (type I fusion) cells were purchased from the American Type Culture Collection. Cells were cultivated on 0.1% gelatin-coated plates (Sigma-Aldrich, Buchs, Switzerland) in RPMI medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine, at 37^{o}C in5% CO2. The PHLDA1b expression plasmids, GFP-PHLDA1b-pEGFP-C1 and PHLDA1b-pcDNA3.1, were a gift of Prof. Dr. Richard Austin (Department of Pathology and Molecular Medicine, McMaster University and the Henderson Research Centre, Hamilton, Canada). Lipofectamine 2000 (Invitrogen) was used to transfect cells according to the manufacturer's protocol. Cell lines with stabile over expression of PHLDA1 were crated upon selection with 800µg/ml G418 for 2 weeks.

Adhesion assay

96-well plates were coated with 15 µg/ml of fibronectin or laminin (Sigma-Aldrich, St. Luis, MO, USA) at room temperature for 1h. They were then washed with PBS and blocked with heat-denatured 1% BSA (HD-BSA). Non-coated wells or wells coated with HD-BSA alone were used as controls. Adhesion assays were carried out with cells grown in tissue culture medium to subconfluency in 10cm Petri dishes. They were then detached with accutase (Sigma-Aldrich), resuspended in medium and seeded at 10^4 cells per well and allowed to adhere at 37°C for 30 minutes. Non-adherent cells were removed by washing with PBS and adherent cells were fixed with 10% formalin in PBS at room temperature (RT) for 15 minutes and then stained with 0.05% crystal violet in H_2O at RT for 15 minutes. Images of randomly selected areas using 4x magnification. The number of adherent cells in the analyzed area was determined with ImageJ software (http://rsb.info.nih.gov/ij/). The total number of adherent cells per well was counted and the percentage of adherent cells was obtained by dividing the number of adherent cells by the

total number of seeded cells and multiplying with 100. The experiments were performed in triplicates and repeated three times.

Proliferation assay and Caspase 3/7 assay

About $5x10^3$ cells were plated in 96 well plate and growth was monitored by making measurements every 24h using WST-1 reagent (Roche) according to the manufacturer's protocol. In the case of Caspase 3/7 activity, $2x10^4$ cells were plated in 96 well plate and 24h later cells were treated with either midostaurin (provided by Novartis), fenretinide (Sigma Aldrich) or DMSO. After 24h cells were lysed and assayed for Caspase 3/7 activity using CaspaseGlo kit (Promega) according to the vendor's protocol.

Human ES tissue microarray analysis

ES tissue specimens were collected from 34 patients during primary tumor resection in accordance with the regulations of the local ethic committee. Clinical data of the patients are presented in Table 1. Microarray sections of 4.5 µm were processed as reported[236] and stained with a monoclonal PHLDA1 antibody (Sigma Aldrich) (1:100) and counterstained with hematoxylin. Tissue microarray grading was performed based on the intensity. The intensity of the stain was judged by eye (negative, poor positive, moderate positive, strong positive).

Results

Based on the previous knowledge regarding the role of PHLDA1, especially implications of its putative tumor suppressor role in melanoma and breast cancer, we decided to perform a set of functional assays. For this reason we transfected two ES cell lines, namely A673 and RDES with PHLDA1 expression vector and under neomycin selection we established stabile cell lines (Figure 1).

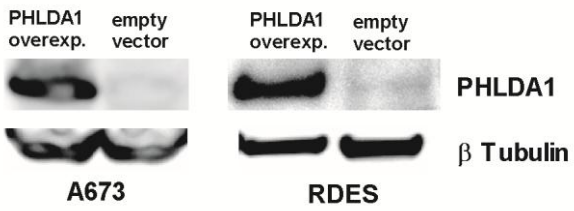


Figure 1 Protein expression of PHLDA1 in two ES stabile transfected cell lines

In order to check if there is any influence on the cell growth we plated these stabile cell lines and performed proliferation assay. As shown in the Figure 2 there was mild effect on cell proliferation. Despite the tendency towards slower growth in cells with over expression of PHLDA1 this effect was not significant.

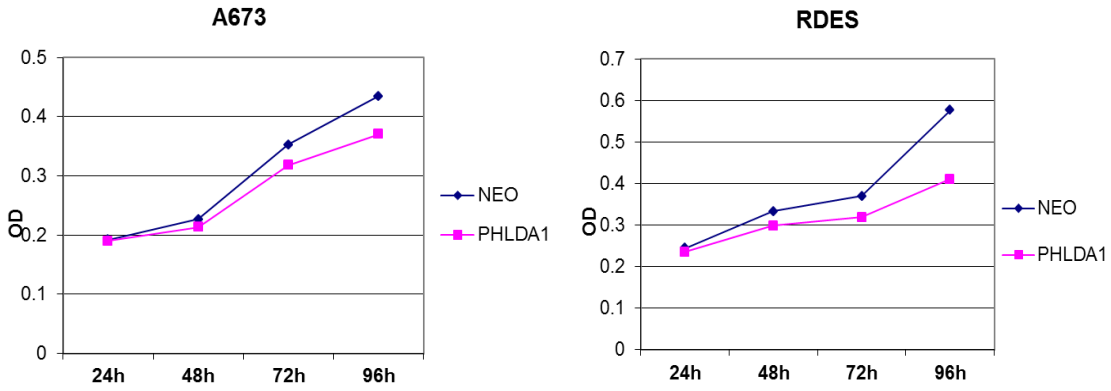


Figure 2 Proliferation of PHLDA1 over expressing ES cell lines: left panel proliferation of A673 cells, right panel proliferation of RDES cells; PHLDA1- stabile cell line overexpressing PHLDA1, NEO- stabile cell line transfected with empty vector, OD- optical density

Given the fact that PHLDA1 was connected to higher levels of basal apoptosis in some human cells, we realized that the approach with stabile cell lines might not be the best, since it is possible that during the selection process we have selected for survivors and hence won't be able to observe any influence on apoptosis. Therefore, we decided to work in parallel with cells transiently transfected with PHLDA1-GFP expressing vectors. In this way we would be able to monitor destiny of GFP positive cells in real time. Interestingly, already 24h after transfection with PHLDA1-GFP expression construct we observed distinct changes in cell morphology. These cells seamed more round, detaching from the plate and in total looked like they are undergoing apoptosis (Figure 3). At the same time, cells transfected with GFP as a negative control did not undergo any morphological changes.

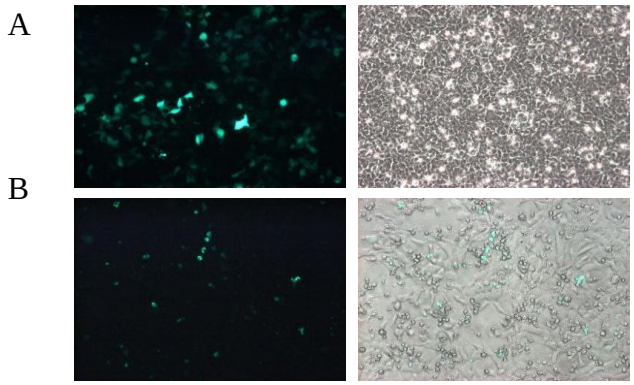


Figure 3 A673 ES cells transiently transfected with GFP (A) or PHLDA1-GFP (B) expression vector

This result led us to hypothesis that PHLDA1 might influence cell adhesion. To test this, we performed short term adhesion assay. Apart from testing adhesion to pre-coated plastic in tissue plates we also coated plates with laminin or fibronectin. As shown in Figure 4, there was a statistically significant reduction in adhesion of PHLDA1 overexpressing cells towards both fully coated plates as well as laminin only coated plates. This was not the case with adhesion to fibronectin. This confirms that PHLDA1 alters binding properties and adhesion of ES cells to extracellular matrix molecules and among these to laminin especially.

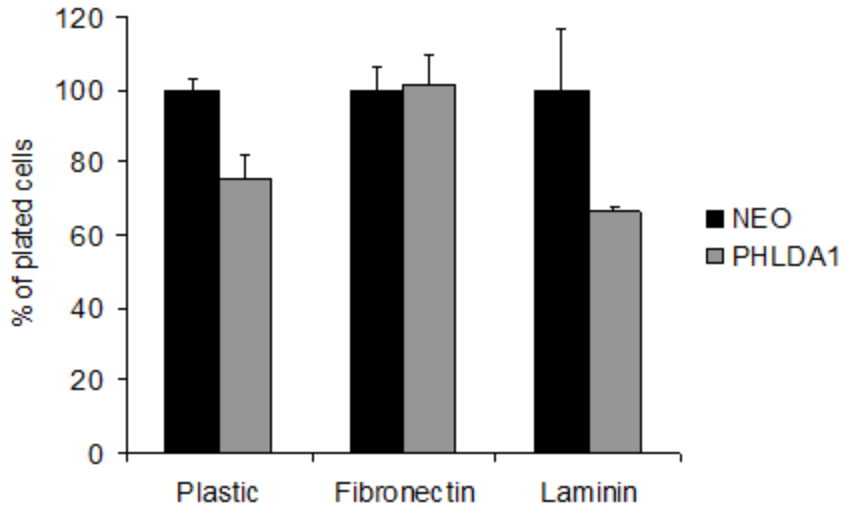


Figure 4 Short term adhesion assay, percentage of adhering A673 ES cells, PHLDA1- stabile cell line overexpressing PHLDA1, NEO- stabile cell line transfected with empty vector

Since PHLDA1 has effect on adhesive properties of the cell we hypothesized that it might play a role in cell detachment mediated apoptosis. We performed caspase assay and measured caspase 3

and 7 activity in A673 cells transiently transfected with PHLDA1-GFP or GFP only expression vectors. As shown in Figure 5, after 48h as well as after 72h there was higher level of basal apoptosis in PHLDA1 over expressing cells. In melanoma cells was shown that higher levels of PHLDA1 expression are correlated to higher sensitivity to treatment with some chemotherapeutics. Therefore, we treated these transiently transfected cells with PKC412 (midostaurin), fenretinide or doxorubicin. We observed that PHLDA1 over expressing cells were more sensitive to PKC412 treatment than the negative cells (Figure 5), while this was not the case with either fenretinide or doxorubicin. Taken together these results imply that PHLDA1 contributes to higher levels of basal apoptosis and at the same time renders cells more prone to midostaurin induced apoptosis.

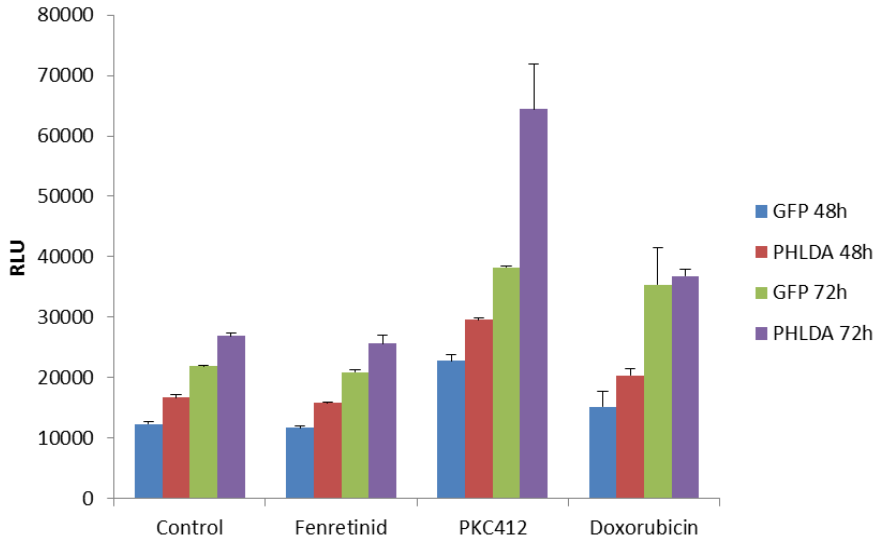


Figure 5 Caspase 3 and 7 activity (in relative luciferase units) of A673 ES cells transiently transfected with GFP or PHLDA1-GFP and treated with PBS, 500nM fenretinide, 500nM PKC412 or 500nM doxorubicine

In the light of the effect of PHLDA1 on cell adhesion, it was interesting to identify a molecular connection. Furthermore, since there was pronounced effect of midostaurin, a broad spectrum kinase inhibitor, we wanted to elucidate connection of PHLDA1 and signaling molecules involved in these processes such as ERK signaling. When cells were stimulated with bFGF we observed an expected increase in phosphorylation of ERK (Figure 6). At the same time PHLDA1 levels increased. Specificity of this effect was shown by using an inhibitor of FGF receptor, namely BGJ398. Along with pERK also PHLDA1 protein level went down upon use of the inhibitor. Thereby we confirmed direct influence of ERK on PHLDA1 levels. Furthermore, we

wanted to check if there is a negative feedback loop, where PHLDA1 would inhibit pERK. When cells were transfected with PHLDA1 expression vector and stimulated with bFGF, there was induction of phosphorylation of ERK but it was less prominent than in the case of non-transfected cells. Also, when cells were transfected with shRNA against PHLDA1, upon stimulation there was steady level of PHLDA1 and hence no negative feedback to pERK that was ultimately reflected by higher level of pERK. Taken together, these results suggest a link between ERK and PHLDA1.

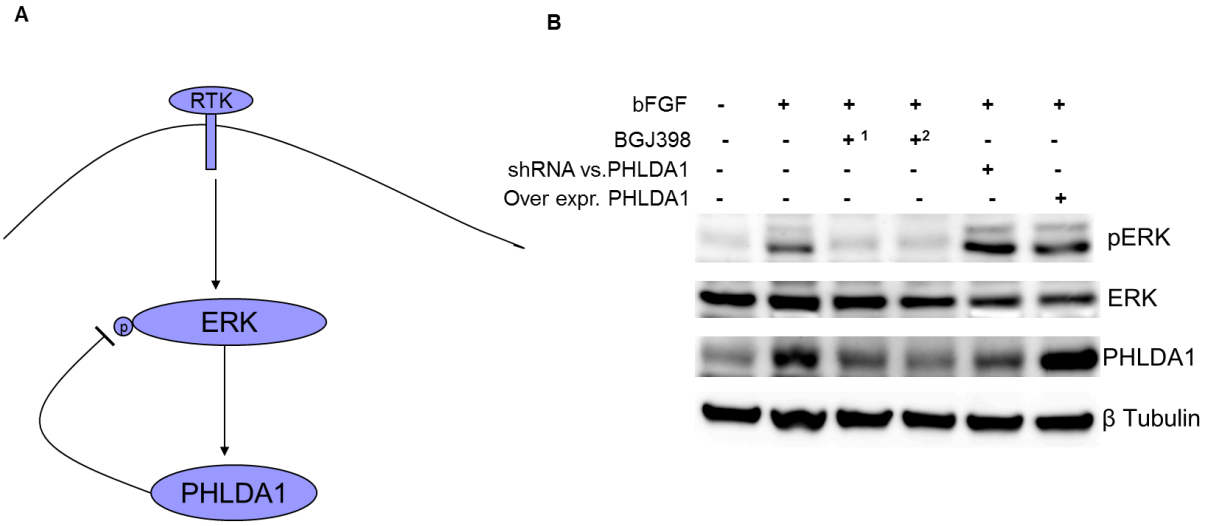


Figure 6 PHLDA1 and ERK signaling in A673 ES cells, (A) Scheme of feedback loop between ERK and PHLDA1, (B) western blot of pERK, total ERK, PHLDA1 and β tubulin as loading control; bFGF- basic fibroblast growth factor, BGJ398- inhibitor of FGF receptor (1- 500nM, 2- 1μM)

Finally, we wanted to check the situation in tumor biopsies from patients. We performed immunohistochemical analysis of TMA containing samples acquired from 34 patients with ES (Table 1). This TMA was stained for PHLDA1 and based on the intensity of the staining, negative, poor positive, moderate positive and strong positive signals were distinguished.

Table 1: IHC analysis of PHLDA1 expression in Ewing sarcoma TMA

Histological type	Total n	Negative	Positive +	Positive++	Positive +++
Ewing's sarcoma	34	25	5	4	0

+ poor positive, ++ moderate positive, +++ strong positive

As shown in Table 1, negative for PHLDA1 were 25, poor positive were 5, moderate positive were 4 and there were no strong positive samples. Week expression of PHLDA1 in tumor sections is therefore in complete accordance with the in vitro data[234]. Due to a small number of tumor samples it was not possible to draw any significant conclusions regarding progression free survival and overall survival. However, it is indicative that PHLDA1 positive tumors were less prone to metastasize (1 out of 9, 11%) in comparison to PHLDA1 negative ones (7 out of 25, 28%). To obtain conclusive data it will be necessary to increase the number of tumor sections analyzed.

Discussion

Understanding ES is tightly related to understanding EWS/FLI1. Being a transcription factor that is crucial for both onset as well as maintenance of the tumor cell it is of outstanding interest to elucidate how is this regulated. It is believed that exactly through deregulated expression of numerous target genes EWS/FLI1 provides cell with the characteristic phenotype. Despite the efforts to pinpoint one or two target genes as the quintessential for ES it is now evident that much more target genes contributes in relatively small portions to what is known as ES. Therefore, starting on our previous discovery that PHLDA1 is repressed by EWS/FLI1 we decided to investigate the functional relevance of PHLDA1 in the context of ES.

PHLDA1 has been shown to regulate apoptosis in vascular endothelial cells triggered by homocysteine[223]. PHLDA1 enhances cell death in neuronal cells, however without Fas induction[237]. Interestingly, PHLDA1 has already been implicated to play a tumor suppressor role in malignant melanomas where down regulation of PHLDA1 expression is associated with the progression of the desease[226]. In breast cancer was shown a direct correlation between amount of PHLDA1 and survival of the patients[231]. These discoveries suggested PHLDA1 as an interesting protein to investigate in cancer research. Having in mind the effect of PHLDA1 in breast cancer cell lines we focused on these very same cellular processes. ES cell lines stably transfected with PHLDA1 did not exhibit significantly impaired growth. However, upon transient transfection with PHLDA1 over expressing vector we observed morphological changes in ES cells, with characteristics of cell detachment induced apoptosis. Since it is known that proteins with plekstrin homology domain are involved in modulation of cellular morphology we assumed that PHLDA1 influences cell adhesion molecules. Therefore, we performed short time adhesion assay

showing that indeed PHLDA1 over expressing cells bind less to laminin while at the same time their binding to fibronectin is not affected. This suggests a possible connection between laminin-binding integrins (α3β1, α6β1, α6β4, α7β1) and PHLDA1 expression. Since one of the major signaling pathways involved in the process of cell adhesion is ERK signaling we focused on finding a link between PHLDA1 and ERK. We were able to show the connection between activated ERK and PHLDA1 expression. Similarly, in breast cancer cells PHLDA1 was shown to be a negative regulator of ERK and opposes ERK-mediated transformation in breast epithelial cells[228].

Since higher levels of PHLDA1 in melanoma cells render them more sensitive to treatment with doxorubicin and camptothecin, we transiently overexpressed PHLDA1 in ES cells. These cells exhibited higher level of basal apoptosis but at the same time they were more sensitive to treatment with midostaurin and were not more sensitive to doxorubicin or fenretinide treatment. If we take into consideration that midostaurin, a broad spectrum kinase inhibitor, induces expression of PHLDA1[234] we conclude that this effect together with overexpression of PHLDA1 probably synergistically induces apoptosis. This fact might be useful for treatment, since only PHLDA1 positive tumors should be sensitive.

Apart from the extensive in vitro data it was interesting to analyze the situation in tumor biopsies. Hence, we performed staining TMA sections for PHLDA1 expression and subsequent analysis. Despite the fact that we had very small number of tumors in TMA, only 34, the result was corroborating previous finding that PHLDA1 is weakly expressed in vast majority of tumors. Too small sample size did not allow to correlate PHLDA1 expression and patient survival. However, it is quite interesting that PHLDA1 expressing tumors metastasized nearly 3 times less than non-expressing ones, consistent with a tumor suppressive role of PHLDA1.

Taken together, these results imply that PHLDA1 as a direct target of EWS/FLI1 has a role in apoptosis, cell detachment and cell adhesion of ES cells. Considering also the indicative TMA results it is tempting to speculate that PHLDA1 has tumor suppressor function in ES. Further studies should substantiate these findings.

6 Discussion

Ewing sarcoma (ES) is the second most frequent bone cancer in childhood. Clinically, ES appears as very aggressive osteolytic tumor with early tendency for development of metastasis. It belongs to the group of small-round-blue-cell tumors and is comprised of largely undifferentiated cells. The unique feature of this tumor is presence of the balanced t(11;22)(q24;q12) translocation in more than 85% of cases[44]. This gene rearrangement results in the expression of a chimeric fusion protein where RNA binding domain of EWS is exchanged by the DNA binding domain of the ets transcription factor FLI1, thus generating a dysregulated transcription factor EWS/FLI1[45].

Extensive evidence indicates that EWS/FLI1 is a hallmark of ES development. Its oncogenic activity is thought to be mediated through inappropriate regulation of target genes that are crucial for the fully malignant phenotype[65, 99-102, 235]. Considering this, identification of target genes regulated by EWS/FLI1 protein represents an important step in understanding the underlying molecular mechanisms. Therefore, identification and characterization of novel EWS/FLI1 target genes was one of the aims of this thesis. Hence, we combined microarray analysis of patient tumor biopsies with cell line tumor models to define new EWS/FLI1 target genes. This resulted in a list of six genes that are candidate targets of the fusion protein. The only gene in this list whose expression is low in ES patient tumors compared to other small blue cell tumors and is induced upon silencing of EWS/FLI1 was PHLDA1, suggesting that PHLDA1 is repressed by the fusion protein. Additional examination of more recently published microarray data revealed PHLDA1 as top-ranking gene in two different studies as well [103-104] thus confirming our analysis. To confirm that PHLDA1 is indeed a EWS/FLI1 target gene we performed detailed analysis of its promoter. Therefore, we performed a series of luciferase assays with various deletion and point mutation constructs of the PHLDA1 promoter. These experiments indicated that EWS/FLI1 represses expression of PHLDA1. Finally, chromatin immunoprecipitation experiment confirmed direct binding of EWS/FLI1 to the promoter of PHLDA1. Knowing that PHLDA1 is repressed by EWS/FLI1 we decided to investigate the functional relevance of PHLDA1 in the context of ES.

PHLDA1 has already been implicated to be a tumor suppressor in malignant melanomas where down regulation of PHLDA1 expression is associated with the progression of the desease[226]. The amount of PHLDA1 was shown to be in a direct correlation with a survival of the patients[231]. All this led us to hypothesis that PHLDA1 might have similar tumor suppressive role in ES as it has in melanoma and breast cancer. Even though ES cell lines stably transfected with PHLDA1 did not exhibit significantly impaired growth we observed morphological changes upon transient

transfection with PHLDA1. These cells displayed characteristics of cell detachment induced apoptosis. Since it is known that proteins with plekstrin homology domain are involved in modulation of cellular morphology we hypothesized potential link between PHLDA1 expression levels and integrins and/or other cell membrane molecules in charge for binding to extracellular matrix. To test that we performed short time adhesion assay showing that indeed PHLDA1 over expressing cells bind less to laminin and at the same time their binding to fibronectin is not affected.

Melanoma cells show sensitivity to treatment with doxorubicin and camptothecin that is in direct correlation with PHLDA1 levels. In ES cells overexpression of PHLDA1 led to increase in level of basal apoptosis. At the same time these cells were more sensitive to treatment with midostaurin. Considering that midostaurin is inducing expression of PHLDA1[234] it is possible that PHLDA1 overexpression and midostaurin act synergistically on apoptosis. Knowing this we assume that PHLDA1 positive tumors would be more sensitive to treatment and this might be useful for the treatment with midostaurin.

Finally, we performed staining TMA sections for PHLDA1 expression and subsequent analysis. Despite the fact that we had very small number of tumors in TMA, only 34, the result was confirming previous finding that PHLDA1 is highly repressed in vast majority of tumors. However, small sample size did not allow us to make correlation of PHLDA1 expression and patient survival. Nevertheless, it is interesting that PHLDA1 expressing tumors metastasized nearly 3 times less than non-expressing ones.

These results imply that PHLDA1 is important target gene of EWS/FLI1 since it plays a role in apoptosis, cell detachment and cell adhesion of ES cells. In vitro data together with TMA results suggests that PHLDA1 has tumor suppressor function in ES.

Pediatric sarcomas are rare and this is the main obstacle for development of novel therapeutic approaches. Even though that expression of oncogenic fusion proteins generated by distinct chromosomal translocations is necessary for both tumor development as well as tumor maintenance, direct targeting of these transcription factors using small molecules is still not possible. Therefore, in this thesis we decided to address this issue.

We established an expression based read-out system to measure modulation of transcriptional activity of EWS/FLI1 for small molecule discovery. Based on measuring expression levels of only few direct target genes, this approach was an important simplification of the previously applied gene expression based high-throughput screening [177]. At the same time this is more targeted approach than screenings based on survival or proliferation only, while it is more robust than a screening approach based on a single-gene reporter assay [238]. To increase the robustness of our approach, we quantitatively measured expression of both activated as well as repressed target genes of EWS/FLI1 since we expected that unspecific killing would result in generally reduced mRNA levels.

From the 1280 compounds screened with the small molecule library, we identified among the top 10 hits well known chemotherapeutic agents such as camptothecin, etoposide, idarubicin and doxorubicin. In this list was also fenretinide which is undergoing clinical trials for neuroblastoma and ES [239-240]. Interestingly, in recently published work of Barretina et al, ES cell lines were most sensitive to topoisomerase I inhibitors (derivatives of captothecin) among the panel of 479 different cell lines thus confirming results of our screening[241]. Altogether, these results emphasize the robustness of our screening approach and suggest that it can be expanded to additional compound and/or targeted inhibitor libraries.

One of the most promising novel compounds identified in our screen was the pan-kinase inhibitor Midostaurin. This is a small-molecule kinase inhibitor derived from Staurosporine that inhibits several kinases[242-243]. Midostaurin is currently undergoing phase II clinical trials for treatment of leukaemias in adults and in children. As the population of patients available for phase I and II clinical trials with pediatric solid tumors is very limited, midostaurin represents a small molecule with already established low toxicity for such trials. In addition, midostaurin was already shown to be effective against another childhood sarcoma, rhabdomyosarcoma [244]. Hence, we further evaluated its effect against a large panel of ES cell lines which responded to midostaurin treatment at the high nM range with massive apoptosis similar to etoposide that is currently used in ES therapy.

Midostaurin is able to reduce cell growth, induce apoptosis and reduce tumor growth *in vivo*. Similarly to rhabdomyosarcoma, ES xenografts showed high sensitivity upon treatment with the kinase inhibitor midostaurin. In rhabdomyosarcoma, midostaurin modulates phosphorylation

status of the translocation PAX3/FKHR thus inhibiting its transcriptional activity [245]. Based on this we might conclude that pan-kinase inhibitors could have a therapeutic potential in ES and maybe even sarcomas in general. Therefore, they should be considered for the future treatment of such childhood tumors.

Complexity of ES is not caused by a single driving event and therefore it is necessary to elucidate all the major contributors to the fully malignant phenotype. Therefore, we had an aim to use our well established screening approach, only this time to screen a small library of targeted inhibitors that are inhibiting different signaling pathways in order to identify critical pathways. As the most prominent pathway that regulates EWS/FLI1 activity/expression we identified the PI3K pathway. This confirmed earlier findings demonstrating that IGF1R signaling via PI3K is a very important axis in ES cells[136, 183-184, 187]. Indeed, in a subgroup of patients inhibition of IGF1R using monoclonal antibodies showed good initial response. IGF1R leads to activation of several signaling pathways, whereas MAPK pathway is crucial for cell proliferation and PI3K pathway for anti-apoptotic effect[246]. There was an improved therapeutic response when anti IGF1R antibodies were combined with PI3K/mTOR inhibitors. However, even with such combined therapy in some patients resistance was observed. This implies that some other signaling pathways are taking over and provide cancer cell survival.

The most potent compound in our hit list was BEZ235, a dual PI3K and mTOR inhibitor. This compound induced a strong decrease of EWS/FLI1 on both RNA and protein level. Surprisingly, very little is known about the regulation of EWS/FLI1 expression and there is only one available study suggesting possible regulatory transcription factors[247]. Previously was shown that inhibition of mTOR by rapamycin decreases EWS/FLI1 protein levels[220]. Therefore, we validated BEZ235 and demonstrated that the effect on the EWS/FLI1 expression is specific. Moreover, by performing a series of luciferase experiments with the EWS/FLI1 promoter we showed for the first time the direct control of EWS/FLI1 promoter and thus its expression by the PI3K pathway. In a series of reporter gene assays we were able to determine the exact regulatory element within the promoter. However, to determine the identity of the transcription factor(s) under PI3K control that is binding in this region additional studies are necessary. Computational analysis of the sequence suggests several potential binding factors, some of them known to be controlled by the PI3K pathway. One of the candidates is NF-κB, transcription factor well known to be controlled by PI3K signaling. Future experiments will elucidate this issue.

Despite the fact that targeting IGF1R/PI3K signaling shows good results in ES[181, 196, 202-203], the mechanism responsible for the efficacy of anti-IGF1R therapy is still not clear. Silencing EWS/FLI1 has stronger pro apoptotic effect than IGF1R antibody which suggests there is a need for more direct targeting of EWS/FLI1 [248]. It is possible that IGFBP3 as target of EWS/FLI1 can induce apoptosis independently of IGF system[103]. Also, some of other EWS/FLI1 target genes might contribute to induction of apoptosis upon silencing EWS/FLI1. Therefore, apoptosis induced by BEZ235 or other IGF1R/PI3K inhibitors might be a result of decrease in EWS/FLI1 expression. Hence, revealing the direct link between PI3K and EWS/FLI1 is of importance, and identity of the responsible transcription factor(s) might provide novel therapeutic opportunities.

The work performed in this thesis opened up several possibilities as future challenges. Our screening of targeted inhibitors pointed out which signaling pathways are important for the biology of ES. We focused on PI3K pathway as the most prominent candidate in our screening. However, there are other pathways whose inhibition resulted in very specific gene expression signature. Moreover, some of these pathways have never before been described in context of ES and it would be interesting to investigate their role in more depth. Given the fact that single pathway inhibition is often not enough due to the cross-talk between pathways and other resistance mechanisms, the future approach might be to simultaneously target two signaling pathways at the same time and thus ensure successful tumor cell targeting. Also, combining hit compounds from our first screening with targeted inhibitors might be an interesting approach. Even though the mechanism of action is not known for some compounds, this should not prevent us from using those compounds. Camptothecin was the top compound in the first screening and its derivative irinothecan is already in clinical trials for treatment of ES. On the other hand BEZ235 is also in clinical trials as single agent. Since both of these mechanistically very different compounds have been shown to reduce EWS/FLI1 levels in this work, it is reasonable to assume that combined use of these two would have synergistic effect that would lead to reduced dosage and hence lower side effects.

In majority of cancer types oncogenic phenotype is driven by transcription factors which therefore represent prime therapeutic targets. However, direct targeting of transcription factors is not easy since they usually do not have enzymatic activity. So far, only handful of transcription factors has been successfully targeted by direct inhibition. Recent example is inhibition of GLI with antagonist GANT61 that leads to reduced expression of its target genes and preferentially

decreased viability of malignant cells in CLL through induction of apoptosis[249]. In sarcomas and leukemias often cancer cell specific gene rearrangements are present that lead to expression of fusion transcription factors. In this case fusion proteins are frequently crucial drivers of the fully malignant phenotype. Moreover, being expressed exclusively in cancer cells makes them even more appealing therapeutic targets since they provide necessary distinction from normal cells. Therefore, many research groups focused on inhibition of protein interactors, since these fusion transcription factors cooperate with other proteins in assembling the final transcription complex on DNA. One successful case of such targeting was demonstrated in MLL-fusion leukemias. Since MLL-fusion proteins are associated with BET family of acetyl-lysine recognizing, chromatin adaptor proteins, it was shown that small molecule inhibitor I-BET51 prevents binding of Bnd3/4 adaptor protein to chromatin thus abolishing activity of MLL-AF4 or MLL-AF9 fusion transcription factor[250].

The presence of fusion transcription factors in many cancers, such as synovial sarcoma, myxoid liposarcoma and rhabdomyosarcoma makes this field even more attractive since a successful targeting strategy in one fusion protein driven tumor type might be extrapolated to the other similar tumor types. Targeted inhibitor of one fusion transcription factor might be effective against other similar fusion transcription factors. This was nicely demonstrated for ET-743, an inhibitor of FUS/CHOP transcription factor in myxoid liposarcoma that was shown to be effective against EWS/FLI1 activity in ES[251]. The screening approach established in this thesis in essence provides proof of principle and thus it might be used in other similar cancers. The advantage of our approach is that it can be used to discover modulators of fusion transcription factor activity/expression irrespective of the mechanism of action. This is very important as it seems extremely difficult to find direct inhibitors. Moreover, screening approach here described might lead to increased knowledge about signaling pathways that are of importance for ES biology and finally to discovery of novel therapeutic targets.

In conclusion, the results presented in this thesis demonstrate that our screening approach can be used for both screening for compounds effective against ES as well as for screening targeted inhibitors. Together this should help to develop novel therapeutic approaches for the treatment of ES.

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8 Acknowledgments

The work presented in this thesis could not have been possible without the support, help and encouragement of many people.

I would like to express my sincere gratitude to Prof. Dr. **Beat Schäfer** for providing me with the opportunity to pursue my PhD in his laboratory, for his continuous support, advice and trust in my work.

I am thankful to my thesis director Prof. Dr. **Roland Wenger** and Prof. Dr. **Martin Pruschy** for kindly accepting to be in my thesis committee and for their scientific suggestions and directions.

Many thanks go to all current and former lab colleagues for their precious help and friendly atmosphere, especially to members of the Ewing's team **Kathya, Florian, David, Franz, Laura and Maria** for their contribution to my research, for being great friends and cheering me up when times were tough and above all for enjoying my every day singing without complains .

I am thankful to all my close-by and far-away friends for nice times out of the lab, especially to **Ana** and **Aleksandar** for their support and for providing me a second home when I needed it most.

I especially thank **Natko** for continuous support and understanding whenever I went through rough times.

I would like to express my deepest gratitude to my father **Vasilije** for his love and faith in me, and to my sisters **Marija** and **Katarina** for their love and encouragement.

Thank you all!

9 Curriculum vitae

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Education

Mar 2008-Sep 2012	**PhD study** at the University of Zurich, Experimental Oncology Department at University Children's Hospital, Zurich, Switzerland within the "Cancer Biology" PhD Program of Life science Zurich Graduate School
Oct 2006-Feb 2007	**MSc** in Molecular Biology and Physiology, Department of Experimental Biomedicine, Faculty of Biology, University of Belgrade, Serbia *Master Thesis Title*: Analysis of mutations of N-ras proto-oncogene in primary and metastatic ovarian tumors
Jun 2011	Clinical Development Course
Jan 2009	Animal Course
Oct 2008	Clinical Cancer Research Course
Apr 2008	Molecular and Cell Biology of Cancer Course
Sep 1996-May 2000	The First Belgrade High School

Presentations

Apr 2011	Annual Meeting of American Association for Cancer Research, Orlando, Florida, USA, Poster presentation
Feb 2011	Brupbacher Symposium, Zurich, Switzerland, Poster Presentation
Nov 2010	Student Retreat of Cancer Biology PhD Program, Switzerland, Talk

Apr 2009	Student Retreat of Cancer Biology PhD Program at Bogis-Bossey, Switzerland, Poster presentation

Publications

1. **Boro A**, Rechfeld F, Schafer BW, Niggli FK. PI3K/AKT pathway modulates transcriptional expression of EWS/FLI1-manuscript ready for submission

2. **Boro A**, Pretre K, Rechfeld F, Thalhammer V, Oesch S, Wachtel M, Schafer BW, Niggli FK. Small-molecule screen identifies modulators of EWS/FLI1 target gene expression and cell survival in Ewing's sarcoma. Int J Cancer 2012.

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